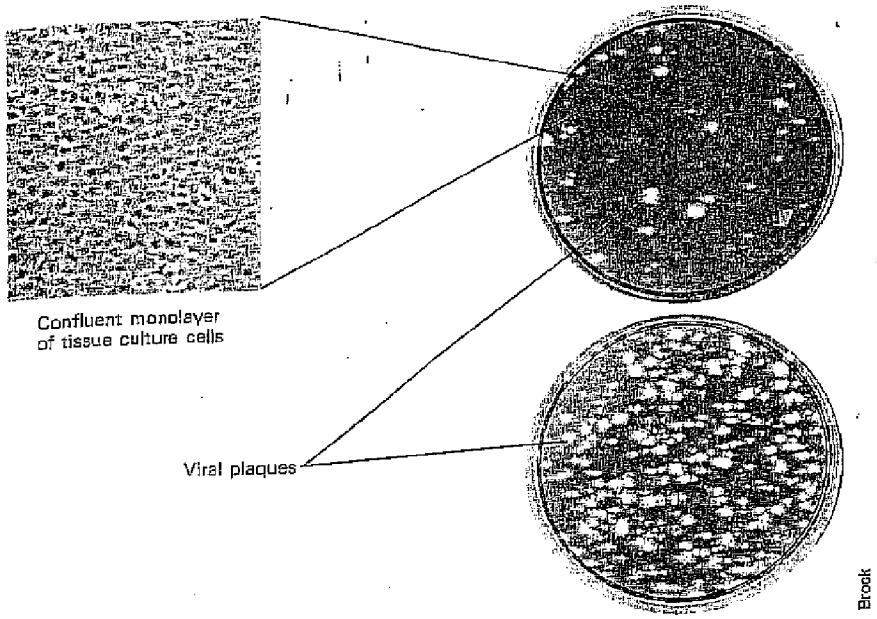

Figure 6.8 Cell cultures in monolayers within petri plates. Note the presence of plaques where virus lysis has occurred. Also shown is a photomicrograph of a cell culture.

Animal infectivity methods

Some viruses do not cause recognizable effects in cell cultures but cause death in the whole animal. In such cases, quantification can only be done by some sort of titration in infected animals. The general procedure is to carry out a serial dilution of the unknown sample, generally at ten-fold dilutions, and samples of each dilution are injected into numbers of sensitive animals. After a suitable incubation period, the fraction of dead and live animals at each dilution is tabulated and an *end point dilution* is calculated. This is the dilution at which, for example, *half* of the injected animals die. Although such serial dilution methods are much more cumbersome and much less accurate than cell culture methods, they may be essential for the study of certain types of viruses.

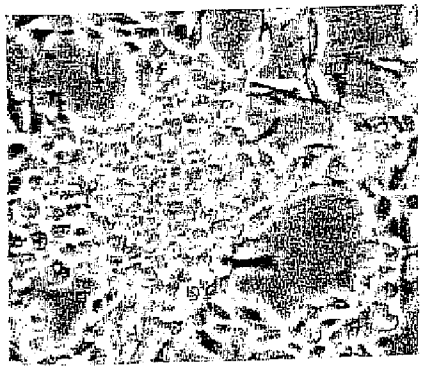

Figure 6.9 Microscopic appearance of a cell culture in which some of the cells have been transformed into tumor cells. The normal cells are elongated because they spread out on the glass culture dish. The tumor cells have lost contact inhibition and have piled up to make a small clump.

Although it requires only a single virus particle to initiate an infectious cycle, not all virus particles are equally infectious. One of the most accurate ways of measuring virus infectivity is by the plaque assay. Plaques are clear zones that develop on layers or lawns of host cells, each plaque due to infection by a single virus particle. The virus plaque is analogous to the bacterial colony.

6.4 General Features of Virus Reproduction

The basic problem of virus replication can be simply put; the virus must somehow induce a living host cell to synthesize all of the essential components needed to make more virus particles. These components must then be assembled into the proper structure and the new virus particles must escape from the cell and infect other cells. The various phases of this replication process in a bacteriophage can be categorized in seven steps (Figure 6.10):

1. **Attachment** (adsorption) of the virion to a susceptible host cell;
2. **Penetration** (injection) of the virion or its nucleic acid into the cell;
3. **Early steps in replication** of the virus nucleic acid, in which the host cell biosynthetic machinery is altered as a prelude to virus nucleic acid synthesis. Virus-specific enzymes may be made;
4. **Replication** of the virus nucleic acid;
5. **Synthesis of protein subunits** of the virus coat;
6. **Assembly** of nucleic acid and protein subunits (and membrane components in enveloped viruses) into new virus particles;

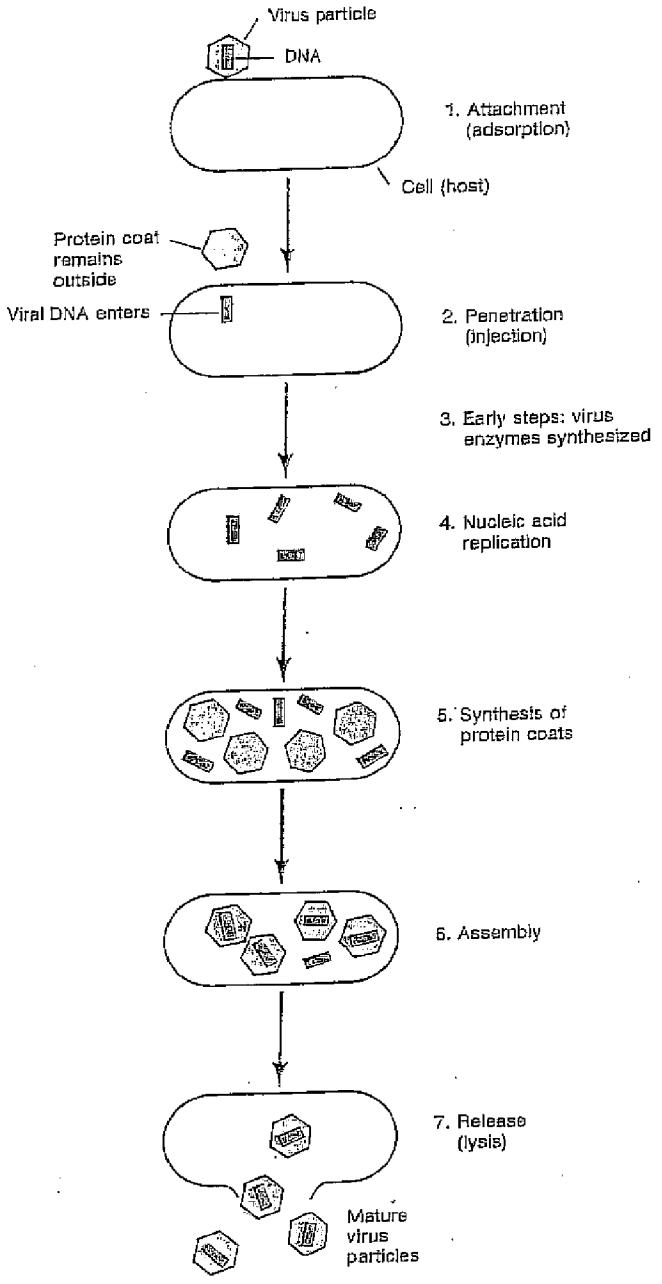

Figure 6.10 The replication cycle of a bacterial virus. The general stages of virus replication are indicated.

7. **Release** of mature virus particles from the cell (lysis).

These stages in virus replication are recognized when virus particles infect cells in culture and are illustrated in Figure 6.11, which exhibits what is called a **one-step growth curve**. In the first few minutes after infection, a period called the *eclipse* occurs, in which the virus nucleic acid has become separated from its protein coat so that the virus particle no longer exists as an infectious entity. Although virus nucleic acid may be infectious, the infectivity of virus nucleic acid is many times lower than that of whole virus particles because the machinery for bringing the virus genome into the cell is lacking. Also, outside the virion the nucleic acid is no longer protected from deleterious activities of the environment as it was when it was inside the protein coat.

The eclipse is the period during which the stages of virus multiplication occur. This is called the *latent period*, because no infectious virus particles are evident. Finally, maturation begins as the newly synthesized nucleic acid molecules become assembled inside protein coats. During the *maturation* phase, the titer of active virus particles inside the cell rises dramatically. At the end of maturation, *release* of mature virus particles occurs, either as a result of cell lysis or because of some budding or excretion process. The number of virus particles released, called the *burst size*, will vary with the particular virus and the particular host cell, and can range from a few to a few thousand. The timing of this overall virus replication cycle varies from 20–30 minutes in many bacterial viruses to 8–40 hours in most animal viruses. We now consider each of the steps of the virus multiplication cycle in more detail.

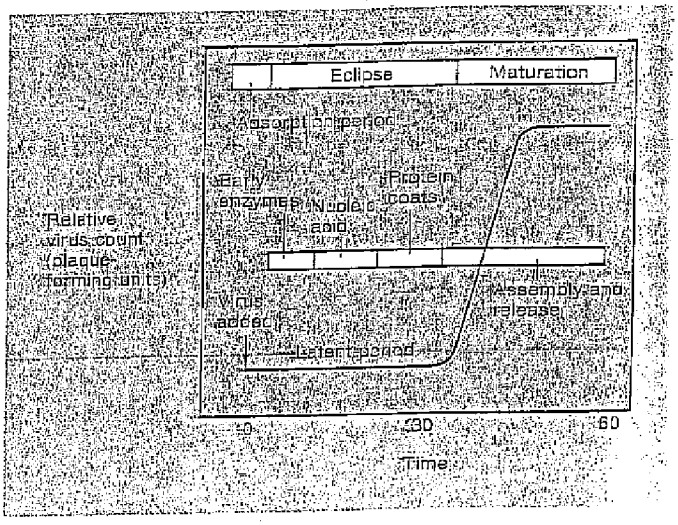

Figure 6.11 The one-step growth curve of virus replication. This graph displays the results of a single round of viral multiplication in a population of cells. Following absorption, the infectivity of the virus particles disappears, a phenomenon called *eclipse*. This is due to the uncoating of the virus particles. During the *latent period*, replication of viral nucleic acid and protein occurs. Then follows the *maturation period*, when virus nucleic acid and protein are assembled into mature virus particles. At this time, if the cells are broken up, active virus can be detected. Finally, *release* occurs, either with or without cell lysis. The timing of the one-step growth cycle varies with the virus and host. With many bacterial viruses, the whole cycle may be complete in 30–60 minutes, whereas with animal viruses 12–24 hours are usually required for a complete cycle.

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CHAPTER 15

Antiviral Therapy

Clyde Crumpacker

One of the remarkable scientific achievements of the past quarter century has been the growth of the infant science of antiviral therapy. There has been a steady stream of effective drugs being developed against an increasing number of viral pathogens. As recently as 1975, it appeared that antiviral therapy would not be possible because animal viruses were such complete obligate intracellular pathogens that inhibition of viral functions would necessarily mean cell death as well. The breakthrough occurred because of the identification of virally encoded enzymes that are essential for virus replication and that differ enough from cellular enzymes to permit selective chemical interactions to occur with viral enzymes but not cellular enzymes. The structural differences between viral and cellular enzymes that have similar functions have permitted chemicals to target viral enzymes and to spare cellular processes. The success of this approach is indeed a surprise. Resistance to antiviral therapy has been a most powerful tool for understanding the mechanism of drug action and the reasons for clinical treatment failure.

In this chapter, the virus groups in which antiviral therapy has succeeded will be described, and the mechanisms of drug action will be outlined. Progress in new antiviral therapies is developing rapidly, and there will be new drugs to inhibit new classes of viruses; these will undoubtedly be developed using principles described here.

HERPESVIRUSES

Historical Background

The first demonstration that antiviral therapy would be successful for treating herpesvirus infections was the use of 5′-iodo-2′-deoxyuridine (IDU) to treat herpetic keratitis. Iododeoxyuridine has significant toxicity in systemic use but was a real advance in the treatment of keratitis caused by herpes simplex virus (HSV), a significant cause of blindness (203). The successful use of IDU proved that herpes infections could respond to antiviral therapy. However, IDU was not a successful therapy in the treatment of HSV encephalitis (122), and its systemic use was associated with significant toxicity. Trifluorothymidine (TFT) is a nucleoside analog that is converted by cellular enzymes to the triphosphate, which inhibits herpes DNA polymerase. It is also used as a topical treatment for herpes keratitis and corneal ulcers (67) and for acyclovir-resistant mucocutaneous human immunodeficiency virus (HIV) lesions (284).

The first antiviral therapy that improved survival in a life-threatening viral disease was adenine arabinoside (Ara-A, or vidarabine), a nucleoside analog of adenine activated by cellular enzymes to form the triphosphate, which is the active inhibitor of the HSV DNA polymerase. Ara-A was administered intravenously to treat

patients with biopsy-proven HSV encephalitis in 1977 (383). Because many other neurologic diseases might present with symptoms similar to those of herpes encephalitis, it was important to use a rigorous diagnostic test to establish a diagnosis. (The ability to culture HSV from temporal lobe brain biopsy tissue was required for enrollment in this study.)

Ara-A also provided effective therapy for other HSV infections, such as neonatal herpes and varicella zoster virus (VZV) infection in the immunocompromised host (380,382). An ointment containing a related compound, Ara-A monophosphate (Ara-Amp), was the first topical ointment to demonstrate an antiviral effect against recurrent herpes simplex labialis (cold sores) (338).

Another nucleoside analog, which possesses strong *in vitro* activity, is bromovinyl deoxyuridine (BVDU), which requires activation by the viral thymidine kinase (TK) to inhibit viral DNA polymerase (84). BVDU is metabolized in humans to bromovinyluridine (BVU), which possesses significant hepatic toxicity, but this drug was never approved for human use. A close relative, bromovinyluridine arabinoside (BVaraU; sorivudine), is much safer for humans; *in vitro* results showed that this nucleoside analog was the most effective inhibitor of the nucleoside analogs tested against human VZV (243). A multicenter clinical trial comparing sorivudine with acyclovir in patients with HIV and herpes zoster showed that the former was more effective for cessation of new vesicle formation and total lesion crusting (147). It was not approved for use in the United States, however, because it enhances the severe toxicity of 5-fluorouracil (5-FU), and the U.S. Food and Drug Administration (FDA) was concerned that the drug would be used in patients receiving 5-FU for cancer treatment.

Acyclovir

Acyclovir [9-(2-hydroxyethoxymethyl)guanine; Zovirax] is a guanosine analog with an acyclic side chain at the nine position and a broken sugar ring. The chemical structure of acyclovir and related drugs for herpesviruses are shown in Figure 1. The structure of deoxyguanosine is included for comparison, to show the position of the broken sugar ring in drugs such as acyclovir, ganciclovir, and penciclovir. Acyclovir is the first truly specific antiviral drug, as it requires activation by a viral enzyme (TK) and has remarkably little toxicity for uninfected cells. Acyclovir is a specific inhibitor of HSV-1, HSV-2, and VZV replication (20,76,109). It is transported into cells either by passive diffusion or by a nucleoside transporter, which also transports guanine in erythrocytes (244). When in the cell, it is readily phosphorylated by the viral TK (see Fig. 1). The viral-encoded TK enzymes of HSV-1, HSV-2, VZV, and Epstein-Barr virus (EBV) catalyze the formation of acyclovir monophosphate (ACVMT), whereas acyclovir is a poor substrate for cellular TK enzymes (114,141,271, 272). This accounts for the remarkable selectivity of acyclovir and the lack of toxicity in uninfected cells (35,271).

In uninfected cells, the cytoplasmic 5′ nucleotidase catalyzes the formation of very low levels of ACVMP (207), but in HSV-infected cells, the level of acyclovir triphosphate (ACVTP) may be 100-fold higher than in uninfected cells, because acyclovir is a much better substrate for the HSV-specific TK and thymidylate synthetase phosphorylating activities (113,138,141,206). A third important area of specificity derives from the fact that acyclovir triphosphate is a highly polar compound that does not easily pass through biologic membranes to exit cells, whereas acyclovir easily enters infected cells and is trapped there in high concentrations. Following its initial phosphorylation, ACVMP is converted by cellular kinases to the acyclovir di- and triphosphates. ACVTP actively inhibits HSV DNA polymerase (139,271,272) and may bind to it 30 times more effectively than to cellular alpha polymerase (97,143). ACVTP competes effectively with deoxyguanosine triphosphate (dGTP) for the viral DNA polymerase and is preferentially incorporated into elongating DNA chains as ACVMP (114,139). The 3′-terminal ACVMP results in chain termination because ACVMP lacks a 3′ hydroxyl group on the 2′ deoxyribose sugar, which is required for a 3′-to-5′ reaction with the next incoming nucleoside triphosphate. A DNA chain that has been terminated with ACVMP is not a substrate for the 3′, 5′ exonuclease activity of the herpesvirus DNA polymerase and thus ACVMP is not excised (92). ACVTP has been described as an *obligate* chain terminator because viral DNA elongation stops completely when ACVMP is incorporated into the elongating viral DNA chain (305). In addition to inhibiting viral chain elongation, ACVTP has also been called a suicide inhibitor because it is an example of an induced substrate inhibitor forming an inactive "dead-end" complex involving viral polymerase, ACVMP-terminated primer template, and the next specific deoxynucleoside triphosphate (dNTP) encoded by the template (305). Cellular DNA polymerases have poor affinity for ACVTP, and very little ACVMP is incorporated into the host cell DNA.

All this accounts for the very low genetic toxicity for host cells associated with acyclovir use (113). The role of ACVMP in chain termination of elongating viral DNA is illustrated in Figure 2. The binding of the next dNTP, which is incorporated immediately following ACVMP, prevents the viral polymerase from disassociating and moving to an alternative extendible primer template. This results in a time-dependent inactivation of HSV-1 DNA polymerase activity (305,306). This mechanism may be selective for the HSV-1 polymerase, as ACTVP does not inhibit the HeLa cell α polymerase (139,304,305). The HSV-1 polymerase is a single polypeptide of 1,235 amino acids. It has been expressed *in vitro*, in yeast, and in insect cells (101,159,249). The cloned protein possesses polymerase activity and can be inhibited by ACVTP in

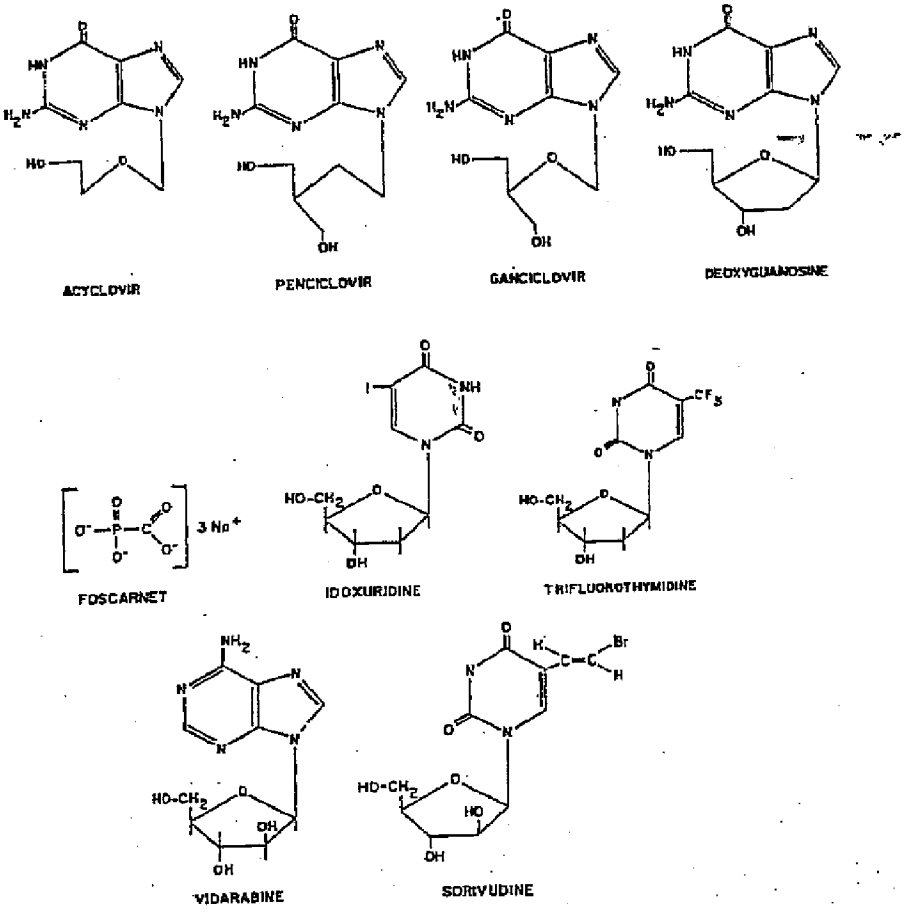

FIG. 1. Chemical structure of antiherpes, antiviral agents, acyclovir (*ACV*), penciclovir (*PCV*), ganciclovir (*GCV*), foscarnet (*PFA*), iodoxuridine (*IDU*), trifluorothymidine (*TFT*), vidarabine (*Ara-A*), and sorivudine (*SVD*). (Reprinted from ref. 172, with permission.)

manner very similar to purified HSV-1 polymerase (101). The functional enzyme consists of a heterodimer involving the HSV-1 DNA polymerase peptide and a smaller polymerase accessory viral protein designated UL42, which enhances polymerase activity and increases processivity of the HSV-1 polymerase (142,152). All herpesviruses probably utilize a similar mechanism—a functionally active polymerase peptide that exhibits greatly enhanced DNA polymerase activity in the presence of a virally encoded polymerase accessory protein. The inhibition of HSV-1 DNA polymerase activity by ACVTP occurs in the absence of the UL42 protein, indicating that ACVTP is able to bind to the polymerase peptide by itself (101).

In summary, the selective action of acyclovir against HSV-1, HSV-2, and VZV is the result of several virus-specific factors: (a) specific phosphorylation by a virus-encoded TK enzyme; (b) specific inhibition of viral DNA polymerase polypeptide by ACVTP acting as a competitive inhibitor with the natural substrate dGTP; (c) chain termination of viral DNA by incorporation of ACVMP, and inactivation of viral DNA polymerase after the incorporation of ACVMP in the presence of dNTPS; and (d) failure of ACVMP and ACVTP to be exported out of cells, with build-up of high levels of intracellular ACVTP. In isolates of HSV-1, HSV-2, VZV, and cytomegalovirus (CMV) from human infections, the relative potencies of inhibition of virus replication in tissue culture are HSV-1 > HSV-2 > VZV, and there is a relative lack of inhibition of CMV replication (Fig. 3) (76). The reason that acyclovir is less active than HSV against VZV is probably related to the short half-life ($t_{1/2}$) of ACVTP in VZV-infected cells, and to VZV's slower replication cycle. The slow replication cycle of VZV would require that a high level of ACVTP be present for a prolonged period, and this is not achieved with the short half-life (2.5 hours) of

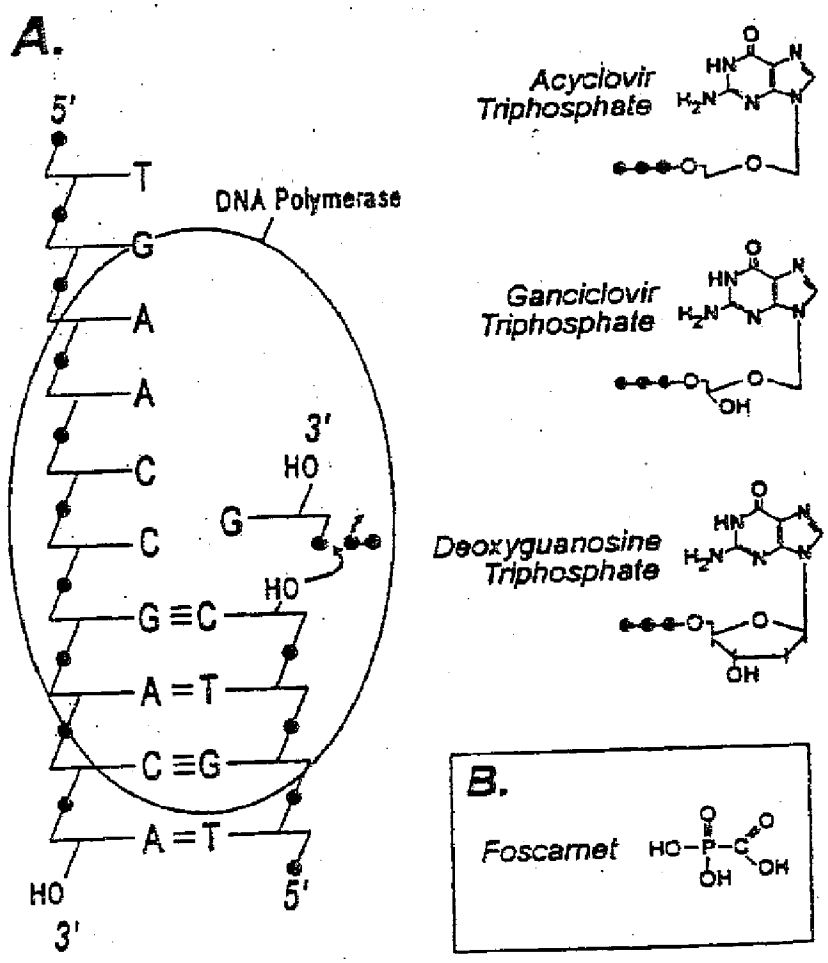

FIG. 2. Inhibitors of the polymerization reaction of herpesvirus DNA polymerase. (Reprinted from Fields BN, Knipe DM, Howley PM, et al., eds. *Fields Virology*, 3rd ed. Philadelphia: Lippincott-Raven, 1996, with permission.)

ACVTP in VZV-infected cell cultures. For comparison, penciclovir triphosphate has a half-life in VZV-infected cells of close to 12 hours. The main reason acyclovir is relatively inactive against CMV is that, because CMV does not possess a virus-encoded TK enzyme analogous to that of HSV, the intracellular concentration of ACVTP achieved in CMV-infected cells is very low. In contrast, ganciclovir triphosphate is specifically phosphorylated through the action of the viral phosphokinase UL97 gene product, and its levels have a prolonged half-life of 16.5 hours in CMV-infected cells (29).

Resistance

Resistance of HSV to acyclovir develops rapidly *in vitro* when HSV is grown in the presence of the drug, and this occurs also in animals and patients receiving acyclovir (75,128,329). The detection of drug-resistance mutations in a specific viral gene has been important to understanding the functions of these genes. Resistance to acyclovir is conferred by mutations in two viral genes, the viral TK and the DNA polymerase genes (55,129,329). The viral TK gene is important for HSV replication in resting or neuronal cells, and TK-deficient mutants of HSV reactivate inefficiently from latency (128,355,356). In many tissues, however, cellular TK enzymes appear to be able to substitute for the viral TK and to support replication of HSV TK mutants.

The most common form of acyclovir-resistant HSV isolate is the TK mutant (35). This may be the result of a single point mutation or a deletion mutant (35,72,212). It has been estimated that about 50% of mutations associ-

of the HIV-1 transmembrane protein gp41. When administered intravenously for 14 days to 16 HIV-infected adults, T-20 exhibited a dose-response effect; a dose of 100 mg twice daily was associated with a median decline in plasma viral RNA of 1.96 $\log_{10}$ (210). Another viral enzyme, viral integrase, is also a potential target of HIV replication inhibition. Compounds that inhibit integrase have been identified but not yet developed into proven drugs (181). Following receptor binding, the virion of HIV is internalized and uncoated, and reverse transcription takes place early in the cytoplasm. Components of RT are packaged and ready to function at once. There is evidence to indicate that reverse transcription begins within the virion (402). In the cytoplasm, in a preintegration complex of viral and probably cellular proteins, the conversion of single-stranded genomic RNA to a double-stranded DNA occurs. The HIV-1 RT catalyzes DNA synthesis and polymerization and also possesses RNase H activity to cleave the single-stranded RNA following initial single-strand DNA formation. The HIV RT, however, lacks an editing function, and errors that arise in the formation of double-stranded DNA are the source of the extensive genetic heterogeneity of HIV.

It is estimated that the mutation rate of this process is 10^{-4}, so an average of one nucleotide base error occurs for every round of reverse transcription (246). The viral preintegration complex is then transported into the nucleus, and the HIV-1 transcribed from proviral DNA is formed after the process of HIV integration. Replication of HIV can proceed following integration into the cellular chromosomal DNA of nondividing terminally differentiated monocyte macrophages and of CD4 T lymphocytes, which are arrested in stages G_2 and S (234,375).

Reverse Transcriptase Inhibitors

The first clinically useful drugs developed for HIV-1 were the nucleoside RT inhibitors (39,139). These drugs first require phosphorylation for the triphosphate to inhibit the HIV-1 RT in a manner analogous to that of most antiherpes drugs.

Phosphorylation is accomplished completely through cellular enzymes, as HIV does not encode any enzyme with similarities to the TK of the HSV. The mechanisms of action have strong similarities to acyclovir, in that the triphosphate is the active inhibitor of HIV-1 RT. Zidovudine (also known as azidothymidine, Retrovir, 3-azido-3-deoxythymidine, or AZT) is a synthetic pyrimidine analog that differs from thymidine in having an azido group at the 3′ position of its deoxyribose ring instead of a hydroxyl group (188) (Fig. 5B). AZT was initially developed as an anticancer drug and was then found to inhibit RT of Friend leukemia virus in 1974 (188,292). In 1985, AZT was also shown to inhibit the RT of HIV-1 *in vitro* (275). The active inhibitor is AZT triphosphate, which is a competitive inhibitor of RT. AZT-TP binds to the HIV-1 RT better than to the natural substrate deoxythymidine triphosphate (dTTP), and it functions as an alternative substrate for the enzyme (137,139,340). AZT-TP possesses a 100-fold greater affinity for the HIV-1 RT than for cellular DNA polymerases alpha or beta (137,139).

This high affinity for HIV-1 RT makes the intracellular concentration of AZT-TP greater than the K_i value for HIV-1 RT but less than the K_i value for cellular DNA polymerase alpha and beta (139). The claim has also been made that AZT-MP is incorporated into growing DNA, and that this leads to premature chain termination (139). HIV-1 RT lacks an exonuclease, so the incorporated nucleoside analog cannot be excised and an incorporated AZT-MP does not posses a 3′-hydroxyl group to form a phosphodiester bond with an incoming nucleotide, so chain elongation is prevented and termination occurs at thymidine residues. The interaction of AZT-TP with the RT produces the selectivity of AZT for inhibiting HIV-1 replication (139,340). The phosphorylation is accomplished by cellular enzymes, and zidovudine is an efficient substrate for cellular TK. Cells exposed to AZT, whether infected or uninfected, accumulate high concentrations of AZT-MP because formation of AZT-DP (the rate-limiting step in the formation of AZT-TP) by host-cell thymidylate kinase is slow. AZT-MP is actually a competitive inhibitor of thymidylate kinase and reduces formation of the natural substrate dTTP. The high intracellular levels of AZT-MP may also impair the RNase H activity of HIV-1 RT (352). The formation of AZT-TP from AZT-DP is catalyzed by the cellular nucleoside diphosphate kinase (139).

As indicated, the phosphorylation of AZT is more efficient in rapidly replicating CD4+ T lymphocytes than in resting monocyte/macrophages. Other drugs may inhibit the phosphorylation of AZT. Ribavirin, a nucleoside analog, is also actively phosphorylated by cellular enzymes. In HIV-infected cells exposed to both zidovudine and ribavirin, the concentration of AZT-TP is diminished and the anti-HIV-1 activity of AZT is reduced (365). Ribavirin, however, increases the antiviral activity of purine analogs such as dideoxyinosine (ddI) by increasing the intracellular concentration of the inhibitor dideoxyadenosine triphosphate (ddATP) and decreasing the intracellular concentration of the natural substrate dATP. These effects of ribavirin are achieved by its inhibition of cellular inosine monophosphate dehydrogenase, which provides a competitive advantage for incorporation to ddATP over the natural substrate dATP (5).

Other nucleoside analogs, including didanosine (2′,3′-dideoxyinosine, ddI), zalcitabine (2′,3′-dideoxycytidine, ddC), lamivudine (3′-thiacytidine, 3TC), stavudine (didehydrodeoxy thymidine, d4T), and abacavir, also have activity against HIV-1 and have been approved for clinical use (167) (Fig. 5B). Didanosine is a prodrug of dideoxyadenosine (ddA) and avoids the reproductive toxicity of orally administered ddA. In the cell, ddI is con-

verted to ddA and the active inhibitor of HIV-1 RT is, in fact, ddA-TP. The mechanism of action for all these nucleoside analogs is similar to that of AZT. Each triphosphate drug functions as a competitive inhibitor of the natural nucleoside triphosphate, and chain termination occurs following incorporation. The ddA, ddC, d4T, and 3TC are phosphorylated by cellular enzymes to the triphosphate and compete with dATP, deoxycytidine triphosphate (dCTP), dTTP, and dCTP (again), respectively, for binding to HIV RT. Abacavir is a guanosine analog and competes with dGTP for incorporation (167).

Resistance

Our knowledge regarding mechanisms of resistance of HIV-1 to RT inhibitors was greatly enhanced by solving the high-resolution crystal structure of this enzyme. The HIV RT is a heterodimer consisting of p51 and p66 subunits. The crystal structure has been determined at a resolution of 3.5 Å. The initial crystal structure was determined on HIV-1 RT complexed to the nonnucleoside RT inhibitor nevirapine, which enabled the enzyme to be stabilized in a conformation that would permit a structure to be determined (216). This was quickly followed by the determination of the HIV-1 RT crystal structure in a complex with a Fab antibody fragment (194). This same research team also determined the crystal structure of many mutant RT molecules. The structure of the HIV-1 RT at 3.2 Å in a complex with the double-stranded DNA template primer and a dNTP has also been described (190).

All of the RT crystal structures show a great deal of agreement, but there are also some contrasts. The structure of the covalently trapped catalytic complex of HIV-1 RT obtained at 3.2 Å, which shows the structure of HIV-1 RT complexed with the DNA template-primer and dNTP, provides a great deal of information regarding mechanisms of resistance and the key role of resistance mutations in RT function (190). The structure of RT containing the Try181Cys mutation and bound to the nonnucleoside RT inhibitor tetrahydrobenzodiazepine (TIBO) has also provided direct evidence of drug binding to an altered RT (82). The p66 subunit of RT contains the active center for dNTP binding, composed of a 183-tyrosine-methionine-aspartate-aspartate (183Y-M-D-D186) region that is absolutely conserved among all RT enzymes in retroviruses and hepatitis B virus (1). It is also conserved in the RNA-dependent RNA polymerase of poliovirus. The p66 subunit has been described as resembling the right hand, whose fingers interact with the position of the template primer, with a palm region and a highly flexible thumb (216). The binding site for the nonnucleoside RT inhibitor nevirapine in the first crystal structure of HIV-1 RT was found in the palm region (216). One of the surprises about the p51 subunit is that although it has the same amino acid sequences as the p66 subunit, its three-dimensional structure is completely different. The p51 subunit appears to function as a scaffold to hold the p66 subunit in place. The catalytic domains of RT are only within the p66 subunit (190,194,216).

The first report of HIV-1 clinical isolates exhibiting resistance to AZT involved patients who had received treatment for at least 6 months. Although the clinical significance of resistance could not be assessed initially (228), HIV-1 also develops resistance to AZT *in vitro* after serial passage of the virus in the presence of the drug (227). The mutations in the HIV-1 RT that confer resistance to AZT were initially reported at codons 67, 70, 215, and 219 (229), and later, mutations at codons 41 and 210 were added (187,205). Mutations at codons 215 and 41 are associated with a high level of phenotypic resistance to AZT and clinical progression of disease (79, 200). The AZT-resistance mutations at codons 67 and 70 are associated with an RT enzyme that possesses increased processivity, whereas the mutations at codons 215 and 219 are associated with mutant enzymes possessing increased rates of pyrophosphorolysis, a reaction that is the reverse of nucleotide incorporation (2). This suggests that chain termination with the incorporated AZT-MP is reversible, and that the AZT-MP can be removed and viral DNA synthesis can be rescued. This appears to be a mechanism for AZT resistance.

Numerous studies to find altered binding of AZT-TP to the mutant enzymes have not conclusively shown it to occur. The rescue of chain-terminated DNA synthesis mediated by ATP or pyrophosphate (PPi) is considered to be an important mechanism that would explain HIV resistance to AZT (2,267). The finger region of the HIV-1 RT is flexible and able to position the incoming RNA template, and many drug-resistant mutations occur as point mutations affecting this domain of the HIV-1 RT. These include the change at K70R and D67N for AZT resistance, the change at K74V for ddI resistance, the change at codon 69 effecting ddC resistance, and the suggestion *in vitro* of codon 75 for d4T resistance (220,253).

Using wild-type RT, the sensitivity to dideoxynucleotides (25) was shown to depend on template length. When the template extension was more than three nucleotides, the wild-type RT began to incorporate dideoxynucleotides. If the RT contained the K74V mutation associated with ddI, then RT did not incorporate dideoxynucleotides, even with a long template extension (25). This clearly suggests a mechanism of resistance involving an altered response to the RNA template. In addition, a series of insertion substitutions, such as a run of serine-serine-serine at codon 69, has been associated with high-level AZT resistance and confers resistance to almost all of the nucleoside analogs (226,387).

An antiviral susceptibility assay that could measure phenotypic resistance to AZT in all the clinical viruses that grow in peripheral blood mononuclear cells was needed to assess the significance of AZT resistance in

Viral Entry Denied

Robert W. Doms, M.D., Ph.D.

Until recently, antiviral drugs were both uncommon and not terribly potent. This has changed: during the past decade, more than 30 antiviral drugs have been licensed, and many of them are very effective. Most of the drugs inhibit the activity of viral enzymes, but a new class of agents that block entry of the virus into the cell is being developed. The development of entry inhibitors is driven by the identification of the cell-surface receptors to which viruses bind and by new findings about viral protein structures that bind receptors and mediate viral entry. These advances offer exciting opportunities for the development of agents that block viral transmission and treat viral infections, as well as for vaccine development.

Most entry inhibitors under clinical development are directed against viruses that are surrounded by a lipid membrane — the so-called enveloped viruses. Regardless of the type of enveloped virus, the fundamental steps of entry are the same. First, the virus attaches to the cell surface, often engaging a specific viral receptor. Viral receptors play a critical role in mediating the entry of the virus into the cell, and the distribution of receptors across specific cell types helps to determine viral tropism. Thus, most strains of the human immunodeficiency virus (HIV) need to engage CD4 and the chemokine receptor CCR5 sequentially to enter the cell, which largely restricts viral infection to certain T cells and macrophages.

Second, binding to the receptor induces the viral-envelope protein to undergo conformational changes that mediate fusion between the viral and cellular membranes (see Figure) in one of two ways. For some viruses, receptor binding leads to endocytosis of the viral particle and delivery to an acidic compartment. There, the low-pH environment triggers conformational changes that lead to membrane fusion. Influenzavirus, West Nile virus, and rabies virus are examples of viruses that use this pathway. For other viruses, the mere process of binding to one or more receptors leads to the needed conformational changes. These pH-independent viruses can fuse at the cell surface; HIV is the best-characterized example.

Since each step of the viral-entry pathway is a potential target for antiviral agents, entry inhibitors fall into several categories, depending on which step they target (see Figure). The first category includes compounds that bind to viral receptors. Small-molecule inhibitors that target the HIV receptor CCR5, which are under clinical development, have been shown to reduce dramatically the levels of circulating virus in HIV-infected patients. Because they target an invariant cellular protein, use of these compounds obviates the difficulty of attempting to target a virus that has considerable genetic variability. The success of these compounds in early clinical trials, coupled with resistance to HIV infection in people who lack CCR5, fueled efforts to identify receptors that are engaged by other viruses. The recent identification of angiotensin-converting enzyme 2 as the receptor for the coronavirus that causes the severe acute respiratory syndrome[1] has already led to the identification of antibodies that prevent receptor binding and may lead to the discovery of small-molecule inhibitors as well.

The second category of entry inhibitors includes compounds that bind to the virus and prevent it from interacting with its receptors. Although some neutralizing antibodies have long been known to operate by this mechanism, small-molecule inhibitors that accomplish the same feat have been developed more recently. Crystallographic studies have helped enormously. For example, an understanding of the structure of picornaviruses (such as human rhinoviruses) has led to the development of a whole series of compounds that fit into a pocket on the viral surface. Some of these compounds block viral attachment, and several have been tested in clinical trials. A small-molecule inhibitor that prevents binding of the HIV envelope protein to the CD4 receptor is also being tested in clinical trials.

The final category of entry inhibitors prevents the conformational changes needed for membrane fusion and includes enfuvirtide, an inhibitor of HIV membrane fusion that has been licensed by the Food and Drug Administration. Enveloped viruses appear to use two classes of membrane-fusion proteins. Class I fusion proteins — such as those found on HIV, influenzavirus, Ebola virus, and respiratory syncytial virus — are trimers of identical subunits

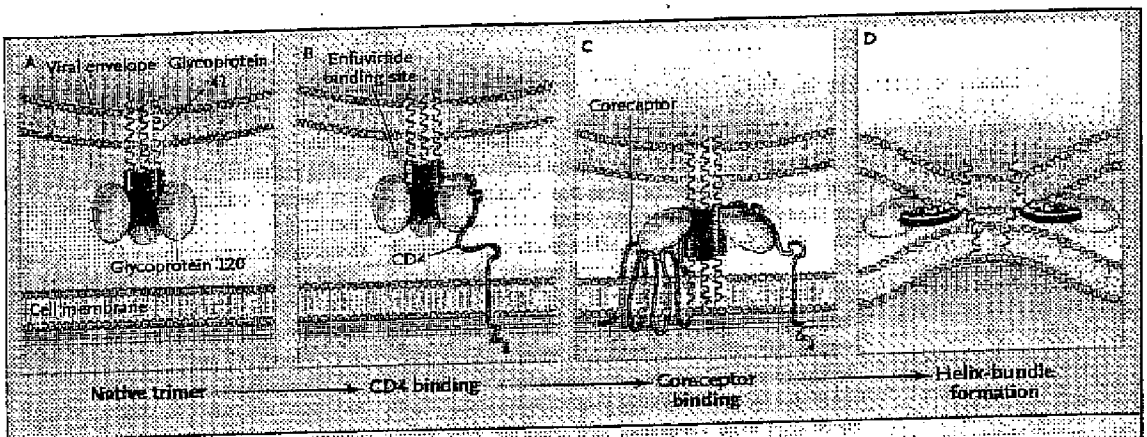

Figure. The HIV-Entry Process as a Model for Class I Fusion Proteins.

The HIV envelope protein is a trimer, with each monomer containing glycoprotein 120 and glycoprotein 41 subunits (Panel A). Thus, there are three glycoprotein 120 and three glycoprotein 41 subunits in the envelope protein trimer. The glycoprotein 41 subunit has an N-terminal hydrophobic domain termed the fusion peptide, followed by two helical regions, helical region 1 (green cylinder) and helical region 2 (blue spiral). The glycoprotein 120 subunit binds to cell-surface CD4 (Panel B), which induces a conformational change in glycoprotein 120 that exposes a domain important for binding to the coreceptor (purple structure) (Panel C). Coreceptor binding is the final trigger for fusion-inducing conformational changes and is thought to release the fusion peptide of glycoprotein 41 (black spiral), which is inserted into the membrane of the cell. Helical regions 1 and 2 of the glycoprotein 41 protein then fold back and bind to each other, bringing the membrane of the cell and the membrane of the virus into very close proximity (Panel D), resulting in membrane fusion and viral entry. (In Panel D, two trimers are shown.) Enfuvirtide, a peptide whose sequence is based on a portion of helical region 2, binds to helical region 1 after it is made accessible by CD4 binding and prevents the formation of the helical bundle that leads to membrane fusion. Small-molecule inhibitors that are being tested in clinical trials can prevent other steps of the viral-entry pathway, including CD4 and coreceptor binding.

that project from the viral surface. On activation by either acid pH or receptor binding, a series of conformational changes occurs. Part of the fusion protein is inserted into the membrane of the cell, linking the viral and cellular membranes. Fusion is then caused by a conformational change in which the two helical regions of the fusion protein fold back on each other, winching the fusion peptide (inserted in the cell membrane) and the membrane-anchoring region of the viral envelope protein (anchored in the viral membrane) toward each other, a process that brings about lipid mixing (see Figure).[2] Enfuvirtide is a peptide that, by binding to one of these helical regions, prevents the conformational change needed for fusion. Structural studies have guided the development of fusion-inhibiting peptides as well as of small-molecule inhibitors for other viruses with class I fusion proteins. Viruses such as the dengue, yellow fever, and West Nile viruses have class II fusion proteins that work in a somewhat different manner, and recent structural studies have suggested ways to inhibit fusion of these viruses as well.[3]

With the licensing of one entry inhibitor and others being tested in clinical trials, these agents have passed the proof-of-principle stage, a benchmark that is testimony to the value of biochemical and structural studies designed to provide the molecular details of how a virus enters a cell.

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VIROLOGY: ON VIRUS ENTRY INTO ANIMAL CELLS

The following points are made by A E Smith and A Helenius (Science 2004, 304:237)

1. Although extremely simple in structure and composition, viruses are masters of camouflage and deception. Devoid of any means of independent locomotion, they disseminate by exploiting cells and organisms. Aided by rodents, insects, and migratory birds, and passed along by global trade and travel, they move around the world with amazing speed. Once they enter the body of a potential host, they can penetrate mucus layers, move through the blood stream, and disperse with the help of motile cells and neuronal pathways.

2. A critical moment occurs when a virus particle reaches a potential host cell and attaches itself to the surface. It must now deliver its capsid and accessory proteins into the cell in a replication-competent form, ideally with minimal damage t the cell and leaving little evidence of its entry for detection by the immune defenses. This is not a trivial problem because cell membranes are impermeable to macromolecules.

3. Viral particles mediate the transfer of the viral genome and accessory proteins from an infected host cell to a non-infected host cell. The task involves packaging the viral genome (RNA or DNA) and accessory proteins, releasing the package from the infected cell, protecting the essential components during extracellular transmission, and delivering them into a new host cell. Many viruses with a DNA genome must enter the nucleus, whereas RNA viruses, with a few exceptions replicate in the cytosol. Overall, viruses use a "Trojan horse" strategy in which the victim assists the intruder. To extract assistance from the host cell, viruses use the detailed "insider information" that they have acquired during millions of years of coevolution with their hosts.

4. In a typical animal virus particle, the viral RNA or DNA is condensed in icosahedral or helical nucleoprotein complexes call capsids. In enveloped viruses, the capsids are surrounded by a lipid bilayer that contains viral spike glycoproteins. In addition, some viruses contain reverse transcriptases, RNA polymerases, kinases, and other proteins that are important during uncoating, replication, or other early intracellular steps.

5. To infect a target cell, a virus particle proceeds through a multistep entry process, during which each step is preprogrammed and tightly regulated in time and space. The entry steps are virus binding to the cell, endocytosis, and nuclear import. Another critical step in the infection process is uncoating, during which the lipid envelope must be shed and the capsids must be at least partially disassembled to expose a replication-competent genome. Once uncoating has occurred, the mobility of the genome within the cell is restricted.

6. In summary: Viruses replicate within living cells and use the cellular machinery for the synthesis of their genome and other components. To gain access, they have evolved a variety of elegant mechanisms to deliver their genes and accessory proteins into the host cell. Many animal viruses take advantage of endocytic pathways and rely on the cell to guide them through a complex entry and uncoating program. In the dialogue between the cell and the intruder, the cell provides critical cues that allow the virus to undergo molecular transformations that lead to successful internalization, intra-cellular transport, and uncoating. (1-5).

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MEDICAL BIOLOGY: ON VIRAL ENTRY INTO HOST CELLS

The following points are made by Robert W. Doms (New Engl. J. Med. 2004 351:743):

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Herpes Simplex Virus Type 1 Entry Is Inhibited by the Cobalt Chelate Complex CTC-96

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The CTC series of cobalt chelates display in vitro and in vivo activity against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). The experiments described here identify the stage in the virus life cycle where CTC-96 acts and demonstrate that the drug inhibits infection of susceptible cells. CTC-96 at 50 μg/ml has no effect on adsorption of virions to Vero cell monolayers. Penetration assays reveal that CTC-96 inhibits entry of the virus independent of gC and cellular entry receptors. This observation was supported by the failure to detect the accumulation of virus-specified proteins and α mRNA transcripts when CTC-96 is present at the onset of infection. Moreover, virion-associated αTIF does not accumulate in the nucleus of cells infected in the presence of CTC-96. CTC-96 targets the initial fusion event between the virus and the cell and also inhibits cell-to-cell spread and syncytium formation. Furthermore, CTC-96 inhibits plaque formation by varicella-zoster virus and vesicular stomatitis virus as efficiently as by HSV-1. Collectively, these experiments suggest that CTC-96 is a broad-spectrum inhibitor of infection by enveloped viruses and that it inhibits HSV-1 infection at the point of membrane fusion independent of the type of virus and cellular receptors present.

Infection by the alphaherpesviruses herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) results in a variety of viral diseases including oral and genital epithelial lesions, encephalitis, and ocular keratitis (15, 16, 20, 96, 103). Among these, herpetic ocular infection is the leading infectious cause of blindness in developed countries (48, 55, 56, 96). Herpesvirus infections are characterized by their ability to establish latency and reactivate from the latent state (80). In immunocompetent and immunocompromised patients herpesvirus infections are among the most frequent causes of viral disease (78, 93, 104). Both primary and recrudescent infections in immunocompromised patients are life threatening (78, 93, 104). Thus, there exists considerable interest in developing treatments for preventing infection and reducing the pathogenesis of primary and recurrent infections by HSV.

Several nucleoside analogs are approved for use in the treatment of herpesvirus infections (e.g., acyclovir, penciclovir, valaciclovir, and famciclovir), and derivatives of these are being developed and/or are undergoing clinical trials (2, 5, 17). These drugs are activated by the HSV thymidine kinase, and thus their primary target is virus DNA synthesis (26, 32). Not surprisingly, drug-resistant strains are appearing with increasing frequency (13, 14, 17, 28, 29, 54, 70, 85). Resistance arises from mutations in the TK gene (18, 27) or mutations in the gene encoding DNA polymerase (13, 14, 47, 70, 85). Therefore, new drugs need to be developed that target other aspects of the virus life cycle in order to find more effective treatments against the existing drug-resistant strains as well as all the known herpesviruses.

The CTC series of cobalt-containing compounds possess anti-inflammatory (105) and antiviral (3, 22, 24, 97) activity. Several CTC complexes have moderate activity in vitro and in vivo against HSV-1 and 2, varicella-zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) (3, 22, 24, 97). However, the data for the inhibitory effects against VZV, CMV, and EBV are anecdotal (22, 24, 97). Previous studies showed that CTC-96 (Fig. 1), a derivative of CTC-23 (3, 22, 24, 97, 105), is the least cytotoxic and most effective of these compounds against HSV-1 and HSV-2 (3, 22). CTC-96 is also effective in inhibiting HSV-1 replication in tissue culture (3). In a rabbit eye model, CTC-96 is able to reduce the corneal surface HSV-1 titer and facilitate recovery from dendritic keratitis (3, 22). It has been suggested that the anti-inflammatory properties of the CTC complexes may aid in recovery from ocular disease (3). However, CTC-96 is not a global virus inhibitor since it is ineffective in the cottontail rabbit papillomavirus model (72).

The antiherpetic activity of the CTC series has been known for many years. However, neither the mechanism by which nor the stage of the virus life cycle at which CTC-96 exerts its inhibitory action on HSV-1 is known. The body of evidence provided here demonstrates that while virus can attach to cells it cannot enter in the presence of CTC-96. The implications of this inhibition for the use of CTC-96 as a tool for analyzing the biology of HSV-1 entry and as an antiviral therapy are discussed. Furthermore, CTC-96 severely reduces the plaquing efficiency of VZV and vesicular stomatitis virus (VSV).

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Grand Island, N.Y.) supplemented with 5% bovine calf serum (BCS; HyClone Laboratories Inc., Logan, Utah). Human fetal lung-Chang (Helf) cells (BioWhittaker, Inc., Walkersville, Md.) were grown in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc.). The Chinese hamster ovary (CHO) cell lines C8 (95), CHO-HveC-1 (34), IEβ8 (67, 95), and IEβ8/HveA (67, 95) (provided by P. Spear, Northwestern University) were maintained as described previously. Unless otherwise indi-

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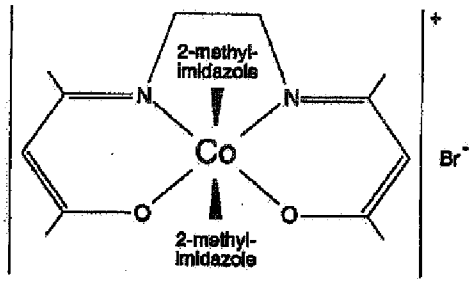

FIG. 1. Chemical structure of CTC-96.

cated, all media used, including overlay media, contained 100 U of penicillin per ml and 100 μg of streptomycin per ml (Gibco BRL).

The wild-type herpesvirus used was HSV-1 Glasgow strain 17 (8). vJSyn$^-$ is described below. vBSΔ27, a *lacZ*-containing ICP27 deletion virus, was described previously (89). The Ellen strain of VZV was provided by P. Annunziato (Columbia University). VSV was provided by V. Racaniello (Columbia University). HS1, a glycoprotein C$^-$ (gC) HSV-1 (KOS) virus, was described previously (73).

HSV-1 preparation. (i) **Cell-associated HSV-1.** Vero cell monolayers were infected at low multiplicities of infection (MOIs) and incubated at 37°C for 2 to 3 days. Infected cells were scraped into the medium and pelleted by low-speed centrifugation. The infected cell pellet was washed with phosphate-buffered saline (PBS) (2.7 mM KCl, 1.2 mM KH_2PO_4, 138 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$), resuspended in DMEM containing 1% BCS, and subjected to five freeze-thaw cycles. The virus was then titrated on Vero cells.

(ii) **Partially purified HSV-1.** Vero cells were infected at a low MOI and incubated at 37°C for 2 to 3 days. Infected cells were scraped into the medium, centrifuged at 900 × *g* for 5 min at 4°C, washed with PBS, and centrifuged again. The infected cell pellet was resuspended in PBS-ABC (PBS containing 5 mM $MgCl_2$ and 7 mM $CaCl_2$) and incubated on ice for 15 min. The cell suspension was disrupted by 15 strokes in a sterile Wheaton Dounce homogenizer using pestle B. The nuclei were pelleted at 3,000 × *g* for 5 min at 4°C. The virions in the supernatant were pelleted by centrifugation at 20,000 × *g* for 75 to 90 min at 4°C, and the virus pellet was resuspended in PBS-ABC-ICS-glu (PBS-ABC containing 1% inactivated BCS and 0.1% glucose). The resuspended virus was pelleted through a 3-ml sucrose cushion (30% sucrose in 50 mM NaCl–10 mM Tris [pH 7.8]) for 2 h at 188,000 × *g* in a Beckman SW41 rotor (6, 25). The virus pellet was resuspended in PBS-ABS-ICS-glu and then titrated on Vero cells.

(iii) **^{35}S-labeled HSV-1.** Vero cells were infected at a MOI of 10. Then 500 μCi of Tran^{35}S-label (1,175 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, Calif.) per ml of methionine-free DMEM (Specialty Media Inc., Lavalette, N.J.) containing 5% dialyzed FBS and 10% normal DMEM (6, 25) was added to the infected cells at 2 h postinfection (p.i.), and the cells were incubated at 37°C for 26 h. ^{35}S-labeled virions were partially purified from the infected cells as described above. The specific activity of the ^{35}S-labeled virus was 5×10^{-4} cpm per PFU.

(iv) **vJSyn$^-$ purification.** Vero cells were infected at a low MOI. Syncytial (syn$^-$) plaques were isolated, and the virus in them was plaque purified two additional times. Cell-associated syn$^-$ virus (vJSyn$^-$) was prepared as described above.

Plaque assays. (i) **HSV-1.** HSV-1 was preincubated with the indicated concentrations of CTC-96 (REDOX Pharmaceutical Corp., Greenvale, N.Y.) on ice for several minutes. The pretreated virus suspension was then diluted to or maintained at the indicated concentrations of CTC-96. For pretreatment of Vero cell monolayers, 50 μg of CTC-96 per ml was added to the medium, and where indicated the cells were then washed in fresh medium with no drug for the indicated times prior to infection with untreated virus. Cells were infected in the presence of various concentrations of CTC-96 or in its absence. After 1 h of adsorption at 37°C in DMEM supplemented with 1% BCS with and without CTC-96, methylcellulose overlay medium (DMEM containing 1.5% methylcellulose and 1% BCS) containing the indicated amount of CTC-96 was added to the infected cell monolayers. The plates were incubated at 37°C for several days and fixed with methanol. The cell monolayers were stained with 0.1% crystal violet, and plaques were counted.

(ii) **VSV.** Virus was diluted in PBS with 0.2% BCS and adsorbed to Vero cell monolayers as described above. After adsorption, infected cells were overlaid with methylcellulose overlay medium and incubated at 37°C for 2 days. Monolayers were fixed and stained as described above for HSV-1.

Adsorption assay. Adsorption of ^{35}S-labeled HSV-1 and detection of bound virus were performed as described previously (6). Briefly, ^{35}S-labeled HSV-1 was adsorbed for 1 h at 4°C to Vero cell monolayers at a MOI of 0.1, 1, or 10 in DMEM containing 1% BCS with or without 50 μg of CTC-96 per ml. The plates were washed four times with 500 μl of ice-cold PBS at 4°C on ice. Each plate was incubated for 10 min in 500 μl of ice-cold radioimmunoprecipitation assay buffer (10 mM $NaPO_4$ [pH 7.2], 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM NaF, 2 mM EDTA) at 4°C. The cpm associated with 100 μl of each lysate was measured by liquid scintillation spectrometry (Wallac Inc., Gaithersburg, Md.). Experiments for each condition were performed in triplicate.

RT-PCR. RNAs were extracted from infected Vero cell monolayers, and the accumulation of α4 and α27 mRNAs was determined by coupled reverse transcription (RT) and PCR using the commercial kit, EZ *rTth* RNA PCR kit (Perkin-Elmer, Foster City, Calif.). The primers used during RT were 4-2 and ICP27-L-RT (58). Following RT, the secondary primers, 4-1 and ICP27-U-RT, were added for PCR (58). These primers result in amplification of a 100-bp fragment and a 220-bp fragment from the α4 and α27 RNAs, respectively.

Western blot analysis. Vero cell monolayers were infected at a MOI of 5 in the presence or absence of 50 μg of CTC-96 per ml. Protein preparation and Western blot analysis were performed as previously described (57). Immunodetection of proteins was performed using the following antibodies: ICP0, rabbit polyclonal antibody CLU7 (57); ICP27, rabbit polyclonal antibody CLU38 (57); glycoprotein B (gB), rabbit polyclonal antibody R69 (provided by G. Cohen, University of Pennsylvania); and αTIF, rabbit polyclonal antibody anti-VP16 (Clontech Laboratories Inc., Palo Alto, Calif.). The secondary antibodies used were goat anti-rabbit and goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Immunoblots were developed as previously described (58).

αTIF immunofluorescence. Vero cell monolayers seeded onto coverslips were infected at a MOI of 100 on ice at 4°C for 45 min. Prewarmed (37°C) DMEM containing 1% BCS was added to each plate, and the plates were incubated at 37°C for the indicated times and then washed twice with ice-cold PBS on ice. The infected cells were fixed in 3.7% formaldehyde in PBS for 30 min and washed with PBS. The fixed monolayers were permeabilized in −20°C acetone for 10 min, and then the cells were washed sequentially with water and PBS. Fixed cells were incubated for 20 min in 10% normal goat serum (NGS; Roche, Indianapolis, Ind.) in PBS containing 0.1% Tween 20 (PBST) and washed twice with PBST. The coverslips were next incubated for 30 min in PBST containing 1% NGS and a 1:200 dilution of rabbit polyclonal anti-VP16 antibody (Clontech Laboratories, Inc.) and washed six times with PBST. The infected cell monolayers were then incubated for 30 min in PBST containing 1% NGS and a 1:200 dilution of goat anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (FITC; Kirkegaard & Perry Laboratories, Inc.) and washed six times with PBST. The coverslips were then mounted on slides in Biomeda gel/mount solution (Fisher Scientific, Springfield, N.J.) and viewed with a 100× lens of a Zeiss LSM 4100 confocal laser-scanning system attached to a Zeiss Axiovert 100TV inverted microscope. Each image is a composite of 1-μm serial sections.

Penetration assays. (i) **Plaque assays.** HSV-1 or HS1 (a gC$^-$ virus) were diluted in PBS with or without 100 μg of heparin sodium salt (Sigma, St. Louis, Mo.) per ml and/or 50 μg of CTC-96 per ml. The diluted virus was then adsorbed to Vero or A549 cell monolayers for 1 h at 4°C on ice and shifted to 37°C for an additional hour. The cells were washed with PBS, citrate buffer (135 mM NaCl, 10 mM KCl, 40 mM citric acid [pH 3.0]) (39, 45), or 48% (wt/wt) polyethylene glycol (PEG) 8000 in PBS (82, 83). The wash buffer was immediately removed, and the cells were carefully washed twice with PBS. Then the infected monolayers were overlaid with methylcellulose overlay medium and incubated at 37°C for several days. Plaques were detected as described above.

(ii) **Liquid β-galactosidase assays.** Three to four days after seeding, CHO cells were infected with wild-type HSV-1 or vBSΔ27 and washed as described above for the plaque penetration assay. After being washed, the cells were overlaid with the appropriate medium and incubated at 37°C for 30 h as described previously (67, 95). The infected cells were washed twice with PBS and lysed in 1× passive lysis buffer (Promega Corp., Madison, Wis.) containing 10 μg of soybean trypsin inhibitor per ml, 5 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone, and 0.1 mM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone. The protein concentration was measured using protein assay dye reagent (Bio-Rad Laboratories, Hercules, Calif.) as specified by the manufacturer. The β-galactosidase activity in the lysates was measured as described previously (84), and β-galactosidase activity

was calculated using the following equation: [optical density at 420 nm/(milliliters of lysate) (time at 37°C)] × 1,000.

Cell-to-cell spread and syncytium formation. Vero cell monolayers were seeded onto coverslips and infected at a MOI of 0.01 in DMEM supplemented with 1% BCS. At 8 h p.i., the medium was replaced with DMEM containing 1% BCS with or without 50 μg of CTC-96 per ml and/or a 1:100 dilution of pooled anti-HSV human sera. At 8 or 16 h p.i., the infected monolayers were washed with PBS, fixed, permeabilized, and examined as described above for αTIF. The primary antibody used was the rabbit polyclonal antibody CLU38 (anti-ICP27) (57), while the secondary antibody was goat anti-rabbit IgG conjugated to rhodamine or FITC (Kirkegaard & Perry Laboratories, Inc.). The slides were viewed with a Leitz Dialux microscope with optical systems for the selective visualization of rhodamine or FITC.

VZV immunohistochemistry. Helf cell monolayers on two-chambered slides were infected with 20 μl of cell-associated VZV in DMEM containing 2% FBS with and without 50 μg of CTC-96 per ml. At 28 h p.i., the slides were washed, fixed, and permeabilized as for αTIF with the exception that the slides were washed two additional times with Tris-buffered saline (TBS) (25 mM Tris, 137 mM NaCl, 3 mM KCl) prior to permeabilization. The slides were then washed twice with TBS, blocked for 20 min in TBS containing 1% goat serum (Sigma), and incubated for 30 min in 200 μl of TBS containing 1% goat serum and a 1:200 dilution of rabbit polyclonal anti-ORF 29 (60). The slides were washed three times with TBS for 5 min each, incubated for 30 min in 200 μl of TBS containing 1% goat serum and a 1:200 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.), and then washed three additional times. The reaction was developed for 5 min using a commercial kit, alkaline phosphatase substrate kit III (Vector Laboratories, Inc., Burlingame, Calif.), as specified by the manufacturer and then washed several times with water. The slides were viewed with a Leitz Dialux microscope.

RESULTS

CTC-96 inhibits HSV-1 replication in tissue culture. The antiviral activity of the CTC complexes against several herpesviruses has been described previously (3, 22, 24, 97). The majority of these studies have been in vivo protocols that addressed the efficacy of the CTC series of compounds against herpesviruses (3, 22, 24, 97). Comparison of several CTC complexes showed that CTC-96 was the most potent inhibitor of HSV-1 in tissue culture and in a rabbit eye model (3). However, the mechanism(s) of action of these drugs is unknown.

Plaque assays were performed to determine the MIC of CTC-96 for HSV-1. CTC-96 at 25 μg/ml prevented the formation of approximately 30% of HSV-1 plaques. By comparison, ≥50 μg of CTC-96 per ml completely inhibited plaque formation (Fig. 2A). This nonlinear inhibitory profile suggests that 25 μg/ml is not sufficient to saturate its target. Furthermore, CTC-96 must be present throughout the initial stages of infection since removal of drug by dilution before adsorption of the virus only partially inhibited the formation of plaques (Fig. 2B). It was unclear whether this partial blockage was the result of a lag in initiation of infection or if the drug affected an aspect of the virus and/or cellular machinery necessary for efficient production of HSV-1 plaques. However, prior incubation of Vero cell monolayers for 4 or 8 h with 50 μg of CTC-96 per ml resulted in a marked decrease in virus yield (Fig. 2C). This decrease in yield was partially reversed if the drug-treated cells were washed before infection (Fig. 2C). These results suggest that short-term treatment with CTC-96 does not irreversibly alter the infectivity of HSV-1 virions. Similarly, while cells exposed to drug for 4 h were severely incapacitated for their ability to support virus replication (≥99%), they produced four- to fivefold more virus following reversal of drug (Fig. 2C). We note that extended exposure (>2 to 3 days) to 50 μg of CTC-96 per ml results in cell death.

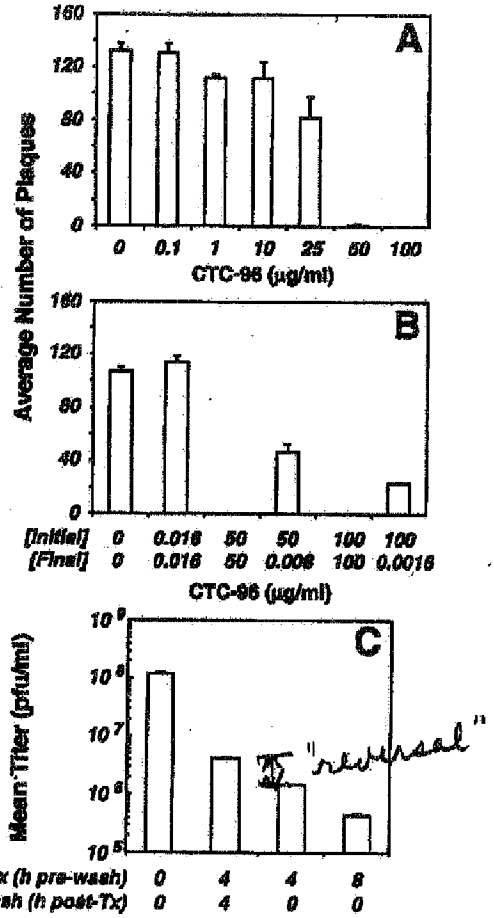

FIG. 2. Effect of CTC-96 on HSV-1 plaque formation in tissue culture. (A) Vero cell monolayers were infected with HSV-1 in the presence of the indicated concentrations of CTC-96. After several days at 37°C, the infected monolayers were fixed and stained and the number of plaques was determined. Data represent the average number of plaques formed from four experiments. (B) HSV-1 was preincubated with the indicated concentrations of CTC-96 [Initial]. Immediately prior to adsorption, CTC-96 was diluted to the indicated final concentrations [Final]. Plaque assays were performed as described in panel A. Data represent the average number of plaques from two experiments. (C) Vero cell monolayers were preincubated with 50 μg of CTC-96 per ml for the indicated times (Tx). CTC-96 was removed, and fresh medium was added to the cells for the indicated times (Wash). The cells were infected with untreated HSV-1, and virus yields were determined 16 h p.i. A representative experiment performed in duplicate is shown.

CTC-96 has no effect on attachment of HSV-1 to Vero cell monolayers. CTC-96 inhibits HSV-1 plaque formation (Fig. 2). However, it is not apparent by what mechanism(s) it achieves this inhibition. A prerequisite for HSV-1 infection is binding of the virion envelope glycoproteins to cell surface receptors (90–92; also see reference 77 and references therein). For instance, gC and gB bind to heparan sulfate (38, 39, 53, 86, 106) while glycoprotein D (gD) attaches to HveA, a herpesvirus entry mediator receptor (67). Suramin was recently shown to block attachment of HSV virions to their cellular receptors (1);

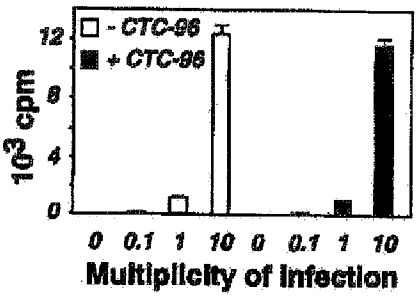

FIG. 3. HSV-1 attachment in the presence of CTC-96. ^{35}S-labeled HSV-1 was adsorbed to Vero cell monolayers at a MOI of 0.1, 1 or 10 on ice for 45 min in the presence (solid bars) or absence (empty bars) of 50 μg/ml of CTC-96. The infected cells were washed several times and the amount of bound virus was quantitated (see Materials and Methods). The data presented are the average cell-associated counts per minute (cpm) and were performed in triplicate.

therefore, it was possible that CTC-96 inhibited binding to the cell surface. Accordingly, we asked whether HSV-1 was able to bind to Vero cells in the presence of 50 μg of CTC-96 per ml. Partially purified ^{35}S-labeled virions were adsorbed to Vero cell monolayers at 4°C, and the amount of radioactivity that remained cell associated after several washes was measured. The amount of virus bound to cells increased linearly with the MOI from 0.1 to 10 PFU per cell and was unaffected by CTC-96 (Fig. 3). Thus, we reasoned that CTC-96 must inhibit a postattachment phase of infection.

Virus proteins do not accumulate when CTC-96 is present during infection. The expression of HSV-1 genes and their gene products occurs in a temporal fashion and is classified into three kinetic classes (43, 44). The production of proteins from all of these classes is required to produce infectious progeny (43, 44). To determine whether the inhibitory action of CTC-96 on plaque formation results from a delay in the temporal order of HSV-1 infection, we examined the accumulation of virus proteins in the presence of 50 μg of CTC-96 per ml. CTC-96 prevented the accumulation of gene products from all kinetic classes when present from the onset of infection (Fig. 4). The appearance of bands reactive with αTIF and gB antibodies, when CTC-96 was present from the initiation of infection, does not result from de novo synthesis of these β/γ proteins (Fig. 4B) (37, 42). Rather, these bands represent the proteins associated with the infecting virions (Fig. 4B) since both αTIF and gB are present in the virion (11, 61–63, 108). If, however, CTC-96 was added after the initiation of infection, there was little or no effect on the accumulation of the α gene products, ICP0 and ICP27 (Fig. 4A), or on the β/γ gene products, αTIF and gB (Fig. 4B). Thus, once the cascade of protein synthesis was initiated, CTC-96 had no significant effect on accumulation of virus proteins (Fig. 4). These results suggest that CTC-96 exerts its inhibitory effect(s) on the HSV-1 life cycle after attachment but at or before the synthesis of virus-specified proteins.

α mRNAs do not accumulate if CTC-96 is present throughout infection. Initiation of HSV-1 immediate-early gene expression does not require de novo protein synthesis (30, 94, 100). Transcription of the α genes is initiated by the virion-associated protein αTIF in concert with the cellular transcription apparatus (10, 36, 71, 75). The protein products of the α4 and α27 genes are essential for the subsequent transcription of β and γ genes (21, 23, 66, 76, 79). Therefore, using RT-PCR analysis, we examined whether the α4 and α27 mRNAs accumulated in the presence of CTC-96. Neither of these mRNAs was detected when CTC-96 was present from the onset of infection (Fig. 5). However, after a short lag period, α4 and α27 mRNAs began to accumulate if CTC-96 was diluted to 0.83 μg/ml before adsorption (Fig. 5). This finding supports the data in Fig. 2 suggesting that the inhibitory effect(s) of CTC-96 is reversible. The lack of accumulation of α mRNAs in the presence of drug does not distinguish between whether CTC-96 acts at the level of α mRNA transcription or at a stage before transcription of α genes. However, the results of this and the preceding experiment suggest that the site of action of CTC-96 must be before virus DNA is transcribed.

αTIF is absent from the nuclei of cells infected in the presence of CTC-96. After entry and uncoating, the HSV capsid moves through the cytoplasm to the nuclear pores (88). αTIF, a tegument protein, also translocates to the nucleus after associating with host cell factor, a cellular protein (51). In addition, αTIF appears to remain associated with the virus capsid in the cytoplasm (107). We therefore determined whether αTIF is present in the nuclei of cells infected in the presence of CTC-96 since this protein is required for transcription of immediate-early genes such as α27 and α4. Indirect immunofluorescence analysis of untreated cells showed that αTIF could be detected in the nucleus by 30 min postinfection (Fig. 6B). Nuclear accumulation was not the result of de novo synthesis, since synthesis of αTIF was not detected until 2 h p.i. under these same conditions (data not shown). When CTC-96

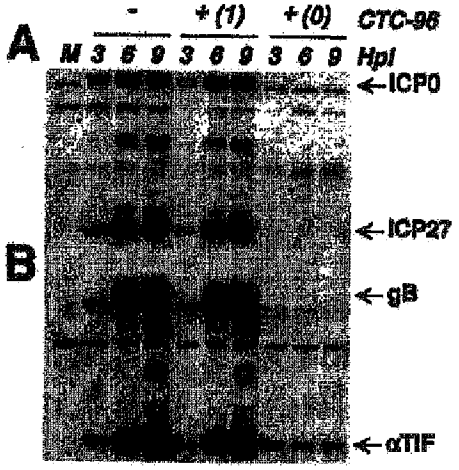

FIG. 4. Accumulation of virus-specified proteins in the presence of CTC-96. Vero cell monolayers were either mock infected (*M*) or infected with HSV-1 at a MOI of 5 in the presence (+) or absence (−) of 50 μgl of CTC-96 per ml. CTC-96 was added either at the onset of infection (*0*) or at 1 h p.i. (*1*). Infected cell extracts were harvested at the indicated hour postinfection (Hpi). Western blot analysis was performed using the rabbit polyclonal antibodies CLU7 (anti-ICP0) and CLU38 (anti-ICP27) (A) or R69 (anti-gB) and anti-VP16 (anti-αTIF) (B).

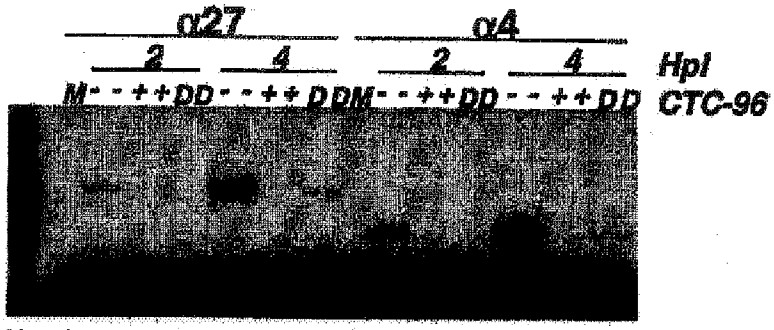

FIG. 5. Effect of CTC-96 on the accumulation of α RNAs in HSV-1-infected cells. Vero cell monolayers were either mock infected (M) or infected with HSV-1 at a MOI of 5 in the presence (+) or absence (−) of 50 μg/ml of CTC-96. HSV-1 pretreated with 50 μg of CTC-96 per ml was diluted (D) to a final CTC-96 concentration of 0.83 μg/ml before adsorption. Total infected cell RNA was prepared at 2 and 4 h p.i. The α4 and α27 mRNAs were amplified by RT-PCR in the presence of [α-^{32}P]dCTP under linear amplification conditions (see Materials and Methods). The amplimers were electrophoretically separated through polyacrylamide gels and visualized by autoradiography. The leftmost lane represents relative size markers. Experiments for each condition were performed in duplicate.

was present, αTIF was not detected in the nucleus at 1 h p.i. (Fig. 6F). In addition, we found that input HSV-1 genomic DNA does not accumulate in the nuclei of cells infected with HSV-1 in the presence of CTC-96 (data not shown). These results lead us to postulate that the introduction of capsid-associated proteins and DNA does not occur in the presence of CTC-96. Thus, while virus can bind in the presence of CTC-96, the drug blocks a step between binding and introduction of the capsid and therefore infection.

CTC-96 inhibits penetration of HSV-1 in a variety of cell types independent of gC and the type of entry receptor. HSV-1 entry is composed of several distinct steps that can be differentiated by their susceptibility to specific washes. The initial stage of attachment involves binding of the viral glycoproteins, gB and/or gC, to cell surface heparan sulfate proteoglycans (38, 39, 86, 106). After binding, the virus becomes resistant to removal from the cell surface by PBS (39–41, 45, 64). However, at this point bound virus is sensitive to elution by heparin and inactivation by low-pH citrate buffer (39–41, 45, 64, 73). After the initial binding event, virus attachment becomes more stable as gD and possibly other virus glycoproteins bind to their cell surface receptor(s). gD binds to several different cellular receptors (HveA to HveD) (12, 35, 50, 67, 99, 102). Binding of gD to its entry receptor appears to play two roles, the first of which is in securing the attachment event and the second is in facilitating or initiating penetration of the virus into the cell. The initial binding of gD to its receptor renders the virus-cell interaction resistant to elution by heparin but sensitive to citrate (39–41, 45, 64, 73). Once the fusion phase of entry is initiated, the bound virions are no longer sensitive to inactivation by citrate buffer (39–41, 45, 64, 73). Penetration assays (39–41, 45, 64) can then be implemented to address the stage affected by CTC-96 by assaying the sensitivity of each phase of entry to specific buffers.

A plaque assay was used to assess whether HSV-1 was able to penetrate cells in the presence of CTC-96 (Fig. 7). To assess whether CTC-96 inhibits entry at a point downstream of the initial gC binding event, a penetration assay using HS1, a gC$^-$ virus, was also performed (73). The absence of gC had no effect on the ability of CTC-96 to inhibit penetration, suggesting that the drug affects an event downstream of gC attachment (Fig. 7B). This result is consistent with the data in Fig. 3 and supports our contention that the block occurs after attachment. It seemed plausible that CTC-96 was having a specific effect on a gD binding or fusion event. Therefore, we assessed whether there was a cell type-dependent difference in the ability of CTC-96 to inhibit virus entry (Fig. 7A and C). CTC-96 inhibited the penetration of wild-type virus in the presence (data not shown) and absence (Fig. 7A and C, right panels) of heparin in the nonhuman primate Vero cell line (Fig. 7A) and in the human cell line A549 (Fig. 7C). Thus, both CTC-96 and heparin, a competitive inhibitor of the first step in virus attachment, block infection.

As described previously, PEG was able to induce fusion of the virus envelope with the plasma membrane (Fig. 7, left and middle panels) (31, 82, 83). However, CTC-96 blocked PEG-mediated fusion (Fig. 7, right panels). While this was surprising, we subsequently demonstrated that CTC-96 also inhibited PEG-induced cell fusion (data not shown). These results suggest that CTC-96 nonspecifically inhibits membrane fusion.

The penetration data do not differentiate between whether the block results from a nonspecific effect on membranes or whether it specifically affects a gD-dependent event. Therefore, to differentiate between these possibilities, penetration assays in CHO cells expressing either HveA or HveC were performed (Fig. 8). CHO cells are inherently resistant to infection by HSV because they lack the appropriate entry receptors (35, 86). Since both HveA and HveC allow entry by most strains of HSV-1 (35, 50, 67, 99, 102), we asked if CTC-96 inhibited entry in CHO cells expressing HveA or HveC. Penetration was assayed by measuring the level of β-galactosidase activity in CHO cells transformed with an αTIF-inducible *lacZ* reporter construct (Fig. 8A and B) or by infection with vBSΔ27, a virus with an αTIF-responsive *lacZ* reporter that does not replicate in noncomplementing cells (Fig. 8C and D). CTC-96 and heparin inhibited HveA-mediated (Fig. 8B) and HveC-mediated (Fig. 8D) entry compared to the no-drug controls (Fig. 8B and D, left panels, respectively) and the untreated CHO cell lines (Fig. 8A and C), which do not express either receptor. Thus, CTC-96 prevents infection of cells and

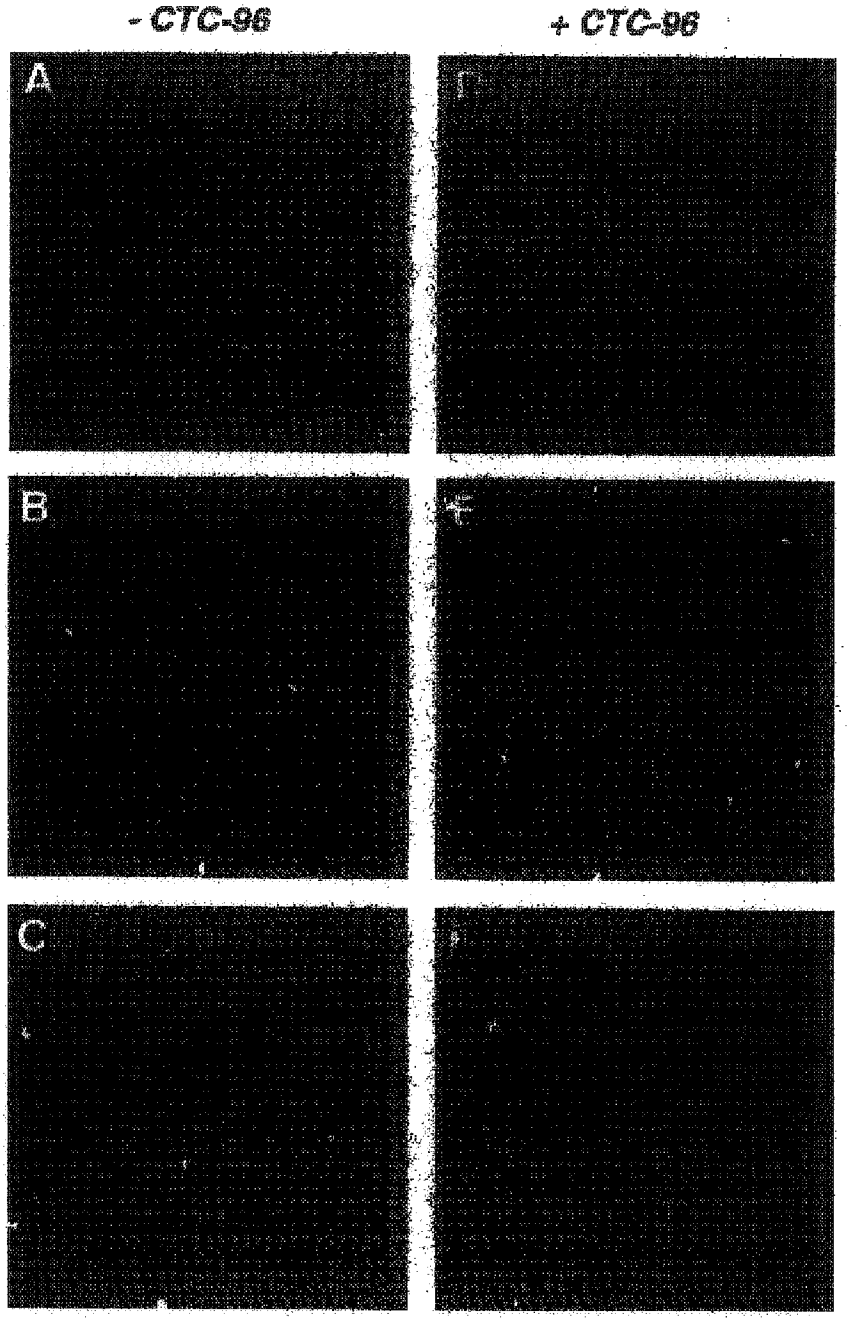

FIG. 6. Accumulation of αTIF in the nucleus of cells infected with HSV-1. HSV-1 was adsorbed to Vero cell monolayers at a MOI of 100 for 45 min at 4°C in the presence (D to F) or absence (A to C) of 50 μg of CTC-96 per ml. Infected monolayers were then warmed to 37°C for 5 min (A and D), 30 min (B and E), and 60 min (C and F), after which they were fixed and permeabilized. The location of αTIF was ascertained using a rabbit polyclonal antibody to αTIF and a goat anti-rabbit IgG antibody conjugated to FITC. The immunofluorescence signal was visualized by confocal microscopy. Each image is a composite of 1-μm serial sections.

the block to HSV-1 entry is independent of the type of entry receptor used.

CTC-96 blocks cell-to-cell spread of wild-type and *syn*$^-$ HSV-1. CTC-96 prevents virus macromolecular synthesis (Fig. 4 and 5), the appearance of αTIF (Fig. 6) and the virus genome in the nuclei of infected cells, and the activation of reporter constructs in CHO cells genetically engineered to be infected by HSV-1 (Fig. 8). The virus glycoproteins (gB, gD, and gH-

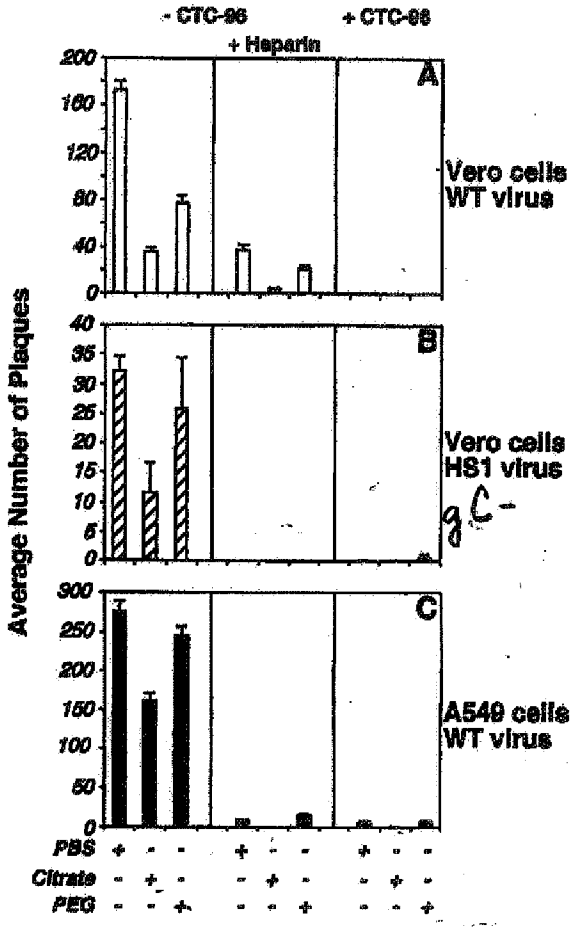

FIG. 7. Effect of CTC-96 on penetration of HSV-1 into monkey and human cells. Vero (A and B) or A549 (C) monolayers were infected with wild-type (WT) (A and C) or gC⁻ (HS1) (B) HSV-1 on ice for 1 h and then shifted to 37°C for an additional 1 h in the presence (+) or absence (−) of 50 μg of CTC-96 per ml and/or 100 μg of heparin per ml. Infected cells were washed with either PBS, citrate buffer, or PEG 8000 as indicated, and infection was monitored by a plaque assay. Although not shown, no plaques were formed on monolayers infected in the presence of CTC-96 and heparin with either type of cells or viruses. Data denote the average number of plaques from one representative experiment performed in triplicate.

gL) and cellular receptors (HveA, HveB, and HveC), that are important for fusion of the HSV-1 envelope with the cell membrane are also involved in virus-induced cell fusion and cell-to-cell spread of virus (9, 19, 68, 69, 95). Therefore, we asked whether CTC-96 was able to inhibit cell-to-cell spread of HSV-1 in tissue culture. Vero cell monolayers were infected at a low MOI in the absence of drug. At 8 h p.i., pooled anti-HSV human sera and/or CTC-96 was added to the infected monolayers. In the presence and absence of neutralizing antibody, HSV-1 was able to spread to adjacent cells, as demonstrated by the spread of the immunofluorescence signal from single cells at 8 h p.i. to surrounding cells at 16 h p.i. (Fig. 9, compare panels A and B). However, after addition of CTC-96 at 8 h p.i., we were unable to demonstrate the development of multicelled foci at 16 h p.i. (Fig. 9C). The ability of CTC-96 to inhibit the spread of wild-type virus was quantified (Table 1). These results demonstrate that CTC-96 added at 8 h p.i. inhibited the spread of virus to adjacent cells. Similar results were obtained with human A549 cells (data not shown).

While cell-to-cell spread and syncytium formation may share some essential factors, previous studies suggest that they are distinct events (12, 35, 77, 90). To determine if CTC-96 inhibits the development of multinucleated foci (syncytia) by syn⁻ viruses, the drug was added at 8 h p.i. and the formation of syncytia was monitored. CTC-96 inhibited the formation of syncytia in Vero cells infected with vJSsyn⁻ (Table 1) and MP (data not shown). These data also demonstrate that the block by CTC-96 occurs in a gK-independent manner as vJSsyn⁻ is defective in gK, and not gB, as determined by its sensitivity to mellitin (4) and cyclosporin A (references 65 and 98 and data not shown). Moreover, when virions labeled in their membranes with the lipophilic fluorescent dye octadecyl rhodamine B chloride are bound to cells in the presence of drug, they fail to dequench (data not shown), supporting our contention that CTC-96 inhibits membrane fusion events. Therefore, CTC-96 inhibits all of the major fusion events in the HSV-1 life cycle, suggesting that it is a nonspecific inhibitor of fusion.

CTC-96 nonspecifically inhibits infection by enveloped viruses. It has been suggested that CTC compounds inhibit infection by other herpesviruses such as VZV, CMV and EBV (22, 24, 97). We confirmed that plaque formation by VZV was inhibited by 50 μg of CTC-96 per ml (Table 2). To ascertain if this inhibition was specific for the herpesvirus family, we asked if 50 μg of CTC-96 per ml inhibited plaque formation by a rhabdovirus, VSV. Table 2 demonstrates that CTC-96 inhibited plaque formation by VSV as efficiently as it inhibited plaque formation by HSV-1 and VZV. Inhibition of VSV plaque formation did not result from a block in endocytosis, since CTC-96 does not inhibit the uptake of Lysotracker, a fluorescent endocytic marker (data not shown). Thus, CTC-96 does not inhibit VSV infection by interfering with endocytosis. These results suggest that CTC-96 inhibits infection by HSV-1, VZV, and VSV, perhaps by targeting a common cellular mechanism that is required for entry by these enveloped viruses.

DISCUSSION

The way in which CTC-96 inhibits HSV-1 infection in tissue culture was studied using assays that probe virus processes that are essential for productive infection. Consistent with a previous report (3), we found that concentrations of CTC-96 of ≥50 μg/ml completely inhibited plaque formation (Fig. 2A and B). Prior incubation of either HSV-1 or cell monolayers with CTC-96 reduced the infectivity of HSV-1 (Fig. 2B) and the ability of cell monolayers to support virus growth (Fig. 2C) in a partially reversible manner. CTC-96 did not affect adsorption of HSV-1 to Vero cell monolayers (Fig. 3). However, because virus does not penetrate cells infected in the presence of drug, no de novo-synthesized virus-specific proteins (Fig. 4), RNA (Fig. 5), or DNA (data not shown) is detected under these conditions. Furthermore, in the presence of CTC-96, virion-associated αTIF (Fig. 6) and HSV-1 DNA (data not shown) also do not accumulate in the nucleus.

Penetration assays demonstrated that CTC-96 was blocking

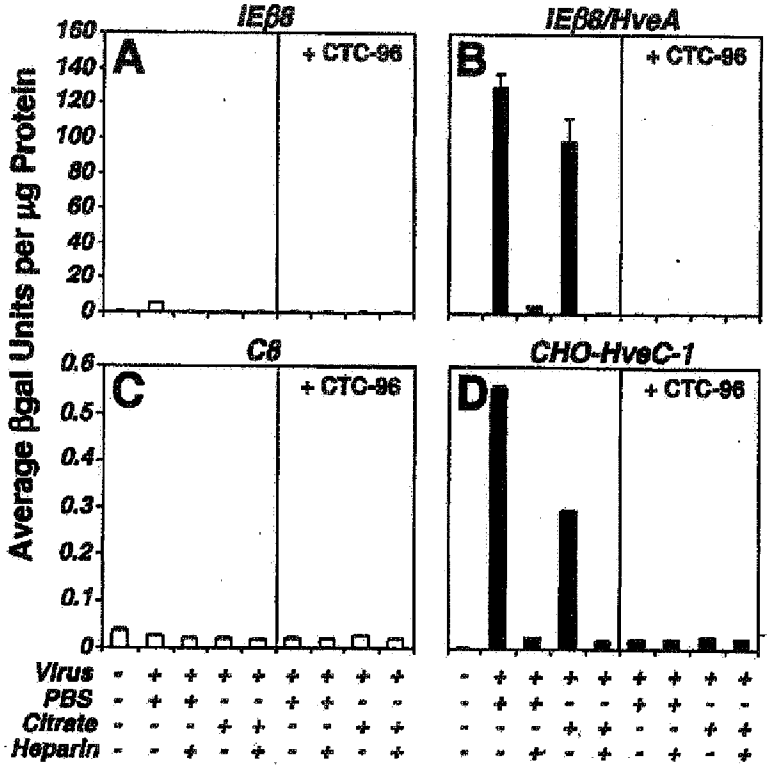

FIG. 8. Assay for virus penetration into CHO cells expressing HveA or HveC. Penetration assays using CHO cells expressing HveA or HveC were performed as in the experiment in Fig. 7, with the exception that penetration was assayed at 30 h p.i. by measuring β-galactosidase (βgal) activity. (A and B) IEβ8 (A) and IEβ8/HveA (B) cells, containing an αTIF-inducible *lacZ* construct, were infected with wild-type HSV-1 virus. (C and D) C8 (C) and CHO-HveC-1 (D) cells were infected with vBSΔ27. Data are presented as the average amount of β-galactosidase activity per microgram of protein of infected cell lysate. Each data set was obtained in triplicate.

virus entry (Fig. 7 and 8). HSV-1 was unable to penetrate either Vero or A549 cells as determined by plaque formation assays (Fig. 7A and C). This effect was independent of gC, since HS1, a gC$^-$ virus, was also inhibited by CTC-96 (Fig. 7B). These observations suggested that an event downstream of attachment was the target for the drug. We therefore assayed the ability of HSV-1 to enter CHO cells expressing either HveA or HveC to determine if this effect was specific for one or more essential gD interactions (Fig. 8). HSV-1 was unable to penetrate CHO cells expressing either entry receptor in the presence of CTC-96 (Fig. 8B and D). Therefore, unlike the antiviral drugs currently approved for treatment of HSV-1, which act on virus-specified enzymes to inhibit replication, CTC-96 prevents the entry of virus into cells.

While our data reveal that CTC-96 inhibits membrane fusion events, they do not provide insight into the exact mechanism of inhibition. CTC-96 may alter the structure of proteins required for membrane fusion by preventing the conformational change of virus glycoproteins and/or cellular receptors that are believed to be important for fusion initiation and completion. CTC compounds selectively unfold proteins in vitro (7). Accordingly, if the cell and virus fusogenic proteins require precise conformations to function, CTC-96 may inhibit their function by preventing protein-protein and/or protein-membrane interactions required for membrane fusion.

Several virus glycoproteins that play a role in the fusion of the virus envelope with the plasma membrane are also involved in cell-to-cell spread and cell fusion. Therefore, to gain further insight into the mode of action of CTC-96, we examined whether virus was able to spread to adjacent cells and/or form syncytia. Addition of CTC-96 to cells previously infected with HSV-1 rendered them unable to form multicelled foci (Fig. 9 and Table 1). Thus, CTC-96 inhibits cell-to-cell spread regardless of whether the virus infects adjacent cells by direct contact with the plasma membranes or via the interstitial space. Furthermore, a syncytium-forming virus, vJSyn$^-$, was unable to form syncytia in the presence of CTC-96 (Table 1). These data do not exclude a specific effect against all the virus and cellular proteins involved in entry, cell-to-cell spread, and virus-induced cell fusion. However, the inhibition of PEG-induced virus-cell (Fig. 7) and cell-cell (data not shown) fusion and the inhibition of infection by other enveloped viruses (Table 2) suggest that CTC-96 exerts its effects nonspecifically. Therefore, the inhibition of the fusion processes involved in HSV-1 infection by CTC-96 suggests that there may be a general mechanism of fusion shared by these processes.

Is there some cellular component also used by adeno + papo (nonenveloped) + both + common + enveloped? or Is

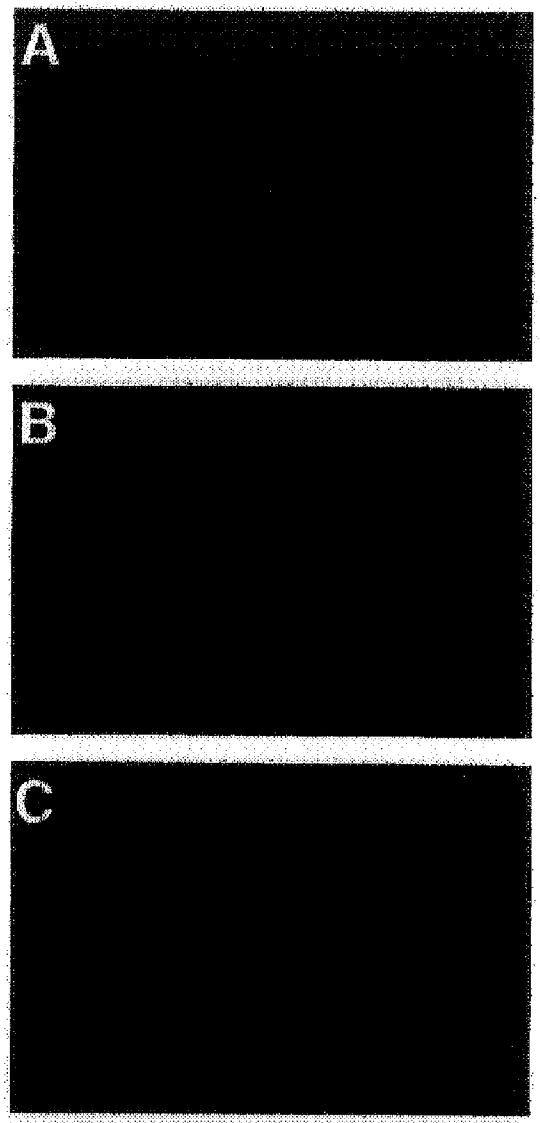

FIG. 9. Cell-to-cell spread of HSV-1 in the presence of CTC-96. Vero cell monolayers were infected at a MOI of 0.01. At 8 h p.i., the medium was replaced with medium supplemented with a 1:100 dilution of pooled human anti-HSV sera with (C) or without (B) 50 μg of CTC-96 per ml. Infected monolayers were fixed at 8 h p.i. (A) or 16 h p.i. (B and C) and stained for ICP27 with a rabbit polyclonal antibody, CLU38, and goat anti-rabbit IgG conjugated to rhodamine.

TABLE 1. The spread of wild-type and syncytium-forming viruses is inhibited by CTC-96

Virus	Time (h) p.i.	HSV antibody	CTC-96 (50 μg/ml)	% of infected cells with HSV-1-positive nuclei	
				Single cell	Plaque[a]
Wild type	8	−	−	91	9
	16	−	−	12	88
	16	+	−	13	87
	16	−	+	84	16
	16	+	+	84	16
vJSsyn$^-$	8	−	−	76	24
	16	−	−	44	56
	16	+	−	48	52
	16	−	+	92	8
	16	+	+	86	14

[a] A plaque is defined as a cluster of cells containing two or more HSV-1-positive nuclei as assayed by indirect immunofluorescence of ICP27.

The CTC compounds were shown to irreversibly bind to and specifically inhibit Sp1, a DNA binding Zn finger protein in vitro (59). Based on this observation, it was postulated that the antiviral activity of the CTC compounds could inhibit human immunodeficiency virus type 1 (HIV-1) by binding to Zn finger containing nucleocapsid proteins as well as to Sp1, which may be important for HIV-1 virus transcription (59). Furthermore, the cytotoxic effects of CTC-96 may result from in vivo inhibition of cellular and viral Zn finger-containing proteins. The HSV-1 immediate-early gene promoters contain numerous Sp1 sites (49) that might provide secondary targets for CTC-96 if it enters cells. However, our results suggest that these are not alternative targets of CTC-96. It seems likely that CTC-96 does not enter cells efficiently because of the temporal requirement for addition of CTC-96 to inhibit virus replication (Fig. 2 and 5). This is further supported by our observations that virus-specified protein synthesis continues unabated when drug is added at 1 h p.i. (Fig. 4) and that there is only a negligible decrease in virus titers when CTC-96 is added at 16 h p.i. and virus is harvested 4 h later (data not shown). Hence, CTC-96 does not appear to act intracellularly. Therefore, based on our demonstration that CTC-96 inhibits virus-mediated cell fusion, the long-term cytotoxic effects of CTC-96 may result from inhibition of global and/or local membrane dynamics, a vital cellular process. Despite its toxic effect on cells in culture, 50 μg of CTC-96 per ml does not appear to be toxic in animal models (3, 22, 24, 97).

We have been unable to isolate CTC-96-resistant viruses. This suggests that either CTC-96 targets one or more essential cellular or virus components or it affects a global process such as membrane dynamics. This latter theory is supported by the demonstration that CTC-96 affects both virus and cellular targets (Fig. 2). Furthermore, this effect may not be specific, since the drug also inhibits PEG-induced cell-to-virus (Fig. 7) and cell-to-cell (data not shown) fusion. If membrane fluidity was altered by CTC-96, it would need to be partially reversible, since infection, which is partially inhibited by preincubation of cells or virus with CTC-96, is restored on dilution of the drug.

TABLE 2. CTC-96 is a broad-spectrum antiviral drug

Virus (strain)	Cell type	% Inhibition[a]
HSV-1 (17)[b]	Vero	100
VZV (Ellen)[c]	Helf	≥98[d]
VSV[b]	Vero	100

[a] Data are shown as percent inhibition compared to the no-drug control.
[b] Data were obtained by plaque assays.
[c] Virus infection was monitored by immunohistochemical analysis using antibody to ORF 29.
[d] Approximately 2% of the drug-treated infected monolayer appeared positive because cell-associated VZV containing cellular debris was used to initiate the infection.

This could reflect de novo synthesis resulting in turnover of the target. Thus, inhibition of virus entry by CTC-96 and its effect on cell viability have implications for a global inhibitory mechanism of membrane coalescence.

The appearance of acyclovir-resistant herpesviruses in patients significantly intensified the effort to develop drugs that inhibit another aspect of the virus life cycle. One attractive target for antiherpetic drugs is the initial stage of infection (i.e., entry and uncoating). Several drugs exist that inhibit HSV infection by blocking attachment or fusion of the virus envelope with the plasma membrane. Heparin, a polysulfonate complex, can block the attachment via gC and or gB through competitive binding for heparan sulfate proteoglycans (64, 86, 106). Sumarin, a derivative of urea, is also able to block HSV attachment but, unlike heparin, is also able to inhibit cell-to-cell spread (1). Unlike heparin and sumarin, *n*-docosanol, a saturated primary alcohol, does not inhibit virus binding; rather, it inhibits fusion of the virus envelope with the plasma membrane of the cell (74). Therefore, it has a broad spectrum and is effective against other enveloped viruses including influenza A virus (74).

In view of a previous report that CTC-96 inhibits infection by other herpesviruses (97), it is likely that the mechanism of inhibition is similar for these viruses. Consistent with previous reports (97), we observed inhibition of VZV and VSV plaque formation by CTC-96 (Table 2). Inhibition of VZV by CTC-96 suggests that the mechanism of action is not specific for gD, since VZV is the only alphaherpesvirus without a known gD homologue (81). Our hypothesis that CTC-96 nonspecifically targets an essential fusion event is further supported by its ability to inhibit plaque formation by VSV. Despite differences in the fusogenic apparatus at the atomic level, it has been proposed that several enveloped viruses share an analogous process of membrane fusion (33, 46, 52, 87, 101). Analysis of the efficacy of CTC-96 against other enveloped viruses could reveal a common mechanism(s) of membrane fusion between viruses and cells.

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